TAMPEREEN TEKNILLINEN YLIOPISTO
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EXPRESSION AND CHARACTERIZATION OF FULL LENGTH INTERFERON GAMMA IN MAMMALIAN CELLS

Master of Science Thesis

Examiner: Prof. Helge Lemmetyinen
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ABSTRACT

Interferon gamma (IFNγ) is an immunomodulatory cytokine hormone, which participates in producing a host defense response to viral and bacterial infections. It has an important role in coordinating expression of immunologically relevant genes overall the human body. Malfunction of IFNγ signaling has been associated with many diseases, for instance deficiencies in resistance to infections such as tuberculosis, and autoimmune diseases including diabetes mellitus and multiple sclerosis.

IFNγ acts via binding to the extracellular domains of its two specific transmembrane receptors, IFNγR1 and IFNγR2, which consequently bind signaling proteins on their intracellular domains, causing a signaling cascade into the nucleus. In this study, IFNγ and its two full length receptors were expressed and purified using protocols modified from previous studies of another transmembrane receptor rhodopsin. The structure of expressed IFNγ and binding of putative antibodies against IFNγ was analyzed using nuclear magnetic resonance (NMR) spectroscopy.

IFNγ and IFNγR1 were successfully expressed transiently in COS-1 cell line, while IFNγR2 was stably expressed in HEK-293 cell line. Full length IFNγR1 and IFNγR2 were found to be expressed as covalently bound dimers with high resistance to reducing conditions. Expressed IFNγ was observed to undergo partial cleavage of glycosylation products and dimerization during storage. Overall, the experimented protocols produced the desired target proteins as heavily glycosylated forms with a high tendency for covalent oligomerization.

The comparison of measured 2D NMR spectrum with an assigned reference spectrum confirmed the identity of the expressed full length IFNγ. One of the examined putative antibodies against IFNγ was observed to bind to the dimer interface and shift the NMR spectrum towards closer correspondence with monomeric IFNγ. Antibody binding is thus expected to change the conformation of IFNγ towards its native state from the observed higher oligomerization states and disturb its signaling function by disrupting the active dimer.

In these experiments, enough protein was expressed for confirming the identity and functionality of IFNγ signaling complex proteins, and for a preliminary study of antibody binding. In order to produce sufficiently protein for studying the interactions of the IFNγ signaling complex in more detail, an inducible stably expressing cell line needs to be established for both receptors and IFNγ. The results of conducted NMR experiments show feasibility of detailed NMR studies of the mechanisms of protein-protein interactions both within the IFNγ signaling complex and with antibodies, in view of drug development.

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TIIVISTELMÄ

Interferon gamma (IFNγ) on immuunijärjestelmän sytokiinihormoni, joka osallistuu kehon puolustusreaktion aikaansaamiseen virus- ja bakteeri-infektioissa. Sillä on myös tärkeä rooli immuunijärjestelmän kannalta olennaisten geenien ilmentymisen säätelyssä kehon eri osissa. Monien sairauksien ilmenemiseen liittyy häiriöitä IFNγ-signaalinvälityksessä. IFNγ:n toimintahäiriö on yhdistetty muun muassa altistumisherkkyyteen tuberkuloosille ja useille muille infektiosairauksille sekä autoimmuunisairauksiin, kuten diabetekseen ja multippeliskleroosiin.

IFNγ vaikuttaa sitoutumalla kahden kalvoreseptorinsa, IFNγR1:n ja IFNγR2:n, solunulkoisiin osiin. Tämän seurauksena reseptorien solunsisäisiin osiin liittyy spesifisiä viestinviejäproteiineja aikaansaaden tumaan johtavan viestinvälitysketjun. Tässä tutkimuksessa IFNγ ja sen molemmat reseptorit ilmennettiin ja puhdistettiin soveltamalla aiemmin tutkitun kalvoreseptorin, rhodopsinin, tuottamiseen käytettyjä menetelmiä. NMR-spektroskopialla suoritettiin tuotetun IFNγ:n rakenneanalyysi sekä tutkittiin sen sitoutumista kehitteillä oleviin IFNγ-vasta-aineisiin.

IFNγ ja IFNγR ilmennettiin tilapäisesti tuottavassa COS-1 solulinjassa, kun taas IFNγR2:lle rakennettiin jatkuvasti tuottava HEK-293 solulinja. Täysmittaiset IFNγR1 ja IFNγR2 ilmenivät kovalenttisesti sitoutuneina dimeereinä, jotka pysyivät sitoutuneina tavallisesti neutraloivissa olosuhteissa. Tuotetussa IFNγ:ssa havaittiin osittaista glykosylaatiotuotteiden pilkkoutumista ja dimerisaatiota säilytettäessä sekä 4 °C että -20 °C lämpötilassa. Sovelletut proteiinien ilmentämis- ja puhdistusmenetelmät tuottivat siis kohdeproteiineja vahvasti glykosyloituneina muotoina, joilla oli taipumus oligomerisoitua kovalenttisesti.

Ilmennetyn IFNγ:n identiteetti vahvistettiin vertaamalla sen mitattua 2D NMR-spektriä tunnettuun referenssispektriin. Yhden tutkituista vasta-aineista havaittiin sitoutuvan dimeerin rajapinnalla sijaitseviin aminohappoihin ja siirtävän NMR-spektriä kohti monomeerisen IFNγ:n spektriä. Vasta-aineen sitoutuminen todennäköisesti muuttaa IFNγ:n konformaatiota ja ehkäisee sen viestinvälitystoimintoa murtamalla aktiivisen dimeerin monomeereiksi.

Tässä tutkimuksessa onnistuttiin tuottamaan riittävästi proteiinia ilmennetyn IFNγ:n identiteetin ja toiminnallisuuden todentamiseksi sekä vasta-aineiden sitoutumisen alustavaan analyysiin. Jotta IFNγ:n signaalinvälitysmekanismien yksityiskohtaiseen tutkimukseen saadaan riittävästi proteiinia, on sekä IFNγ:lle että sen molemmille reseptoreille vakiinnutettava indusoituva jatkuvatuottoinen solulinja. Suoritettujen NMR-kokeiden tulokset osoittavat, että NMR-analyysi on käyttökelpoinen menetelmä sekä INFγ-signaalinvälitysketjun että sitä vastaan kehitettävien vasta-aineiden proteiini–proteiini-vuorovaikutusten tutkimukseen.

PREFACE

The study of Interferon gamma started some thirty years ago, but we are still looking for more efficient methods to express IFNγ and related proteins in full length and in higher yields, in order to discover in more detail the functioning mechanisms of this pharmacologically important hormone. I am glad to have participated in this work and hopefully brought out a little bit of new information that might eventually contribute to the discovery of new drugs or treatments.

The experimental part of this study was conducted in University of Pittsburgh department of Structural Biology, while the written work was finished in Tampere University of Technology department of Chemistry and Bioengineering. I would like to thank Professor Klein for providing the subject, laboratory, materials and guidance for the research, and Professor Lemmetyinen for support in the writing process. I am extremely grateful to Naveena and Balu for their indispensable help in the laboratory, and to Patricia for taking care of my nerves and making me laugh.

A special thank you goes to Ossi for staying up late with me and correcting my commas with love. Finally I want to give credit to the patience and support of my dear parents: Kiitos kärsivällisyydestä isä ja äiti.

In Tampere
1st of August 2012

Nadja Kiiskinen

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Terms and abbreviations

APS
Ammonium persulfate, $(NH_4)_2S_2O_8$, an oxidizing agent which in biochemistry is used together with tetramethylethylenediamine to catalyze the polymerization of acrylamide in preparing gels for protein separation. [1]

BSA
Bovine serum albumin, serum albumin protein derived from cow blood plasma. BSA is used as nutrient in cell and microbial cultures, and as a standard for quantification of proteins because of its signal increasing stability and low cost. [2]

CHAPS
3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, a zwitterionic detergent used in biology to solubilize proteins, especially common in membrane protein purification. [3]

Cytokine
A family of immune system related hormones which act through binding to cell membrane receptors. The activation of membrane receptors initiates a signaling cascade leading to expression of immunologically relevant genes. [4]

DEAE-dextran
Polyethyleneimine, a cationic polymer which assists in cell transfection. The positively charged polymer binds the negatively charged DNA, thus allowing the complex to adsorb to the negatively charged cell membrane and facilitating the intake of DNA by the host cell via endocytosis. [5]

DM
n-Dodecyl-β-D-maltoside, a non-ionic surfactant composed of a maltoside sugar with a long aliphatic side-chain. DM forms micelles which stabilize and solubilize proteins. [6, 7]

DMEM
Dulbecco's Modified Eagle's Medium, a nutritive broth used to maintain cells in tissue culture, originally developed by pathologist Harry Eagle. Contains the essential nutrients, such as amino acids, salts, vitamins and glucose, suitable for most mammalian cell cultures. [7]

DMSO
Dimethyl sulfoxide, $(CH_3)_2SO$, an organosulfur compound which is used as a cryoprotectant for preserving cell suspensions and tissues in long-term storage in liquid nitrogen. [8]

DNA
Deoxyribonucleic acid, a polymeric macromolecule which contains the genetic information in most living organisms (all with the exception of RNA viruses). Consists of a helical double strand of nucleotide units with a sugar backbone bound to phosphate groups by ester bonds. [9]

DTT
(*2S,3S*)-1,4-bis(sulfanyl)butane-2,3-diol, also known as dithiothreitol, a strong reducing agent commonly used to reduce the disulfide bonds in proteins. [10]

EDTA
Ethylene diamine tetra-acetic acid, a preservative that chelates divalent cations such as Mg^{2+} and Ca2+ and so reduces the activity of proteolytic enzymes using these cations as cofactors. EDTA is commonly used in protein analysis to prevent untimely lysis of examined proteins. [8]

FBS
Fetal bovine serum, a supplement rich in growth factors commonly added to cell culture media. [8]

LB medium
Lysogeny broth, a nutritive growth medium for bacterial cells containing peptides, vitamins, trace elements (e.g. nitrogen, sulfur, magnesium) and minerals essential to promote bacterial growth. The common sources used to provide these nutrients are tryptone, yeast extract and NaCl. [11]

KDS
Potassium dodecyl sulfate, an anionic surfactant similar to more commonly used sodium dodecyl sulfate (SDS). [7]

NMR
Nuclear magnetic resonance spectroscopy, a technique based on recording resonance frequencies of atomic nuclei in changing magnetic field. NMR spectroscopy is used to determine structure and dynamics of molecules. [12]

PAGE
Polyacrylamide gel electrophoresis, a method of protein analysis used in to separate different proteins of isoforms of one protein by their size. [13]

PBS
Phosphate Buffered Saline, an aqueous salt solution containing sodium chloride, potassium chloride and sodium phosphates. Used in biological research as a buffer solution to maintain constant pH. [14]

PMSF
Phenylmethylsulfonyl fluoride, a serine protease inhibitor, which binds covalently to the active site serine residue of the enzyme. PMSF is commonly used in solubilizing proteins to inhibit proteases from digesting target protein after cell lysis. [15]

RNA
Ribonucleic acid, a polymeric macromolecule similar to DNA but consisting of only one chain of nucleotides. RNA molecules have multiple functions including regulation of gene expression and protein synthesis. [8]

SDS
Sodium dodecyl sulfate, also called sodium layryl sulfate (SLS), an anionic surfactant which is commonly used in cleaning products and often derived from coconut and palm oils. In biochemistry SDS is used for lysing cells prior to DNA extraction and denaturing proteins for electrophoresis. [7]

Temed
Tetramethylethylenediamine, $(CH_3)_2NCH_2CH_2N(CH_3)_2$, a common reagent in organic and inorganic synthesis. In biochemistry Temed is most often used to catalyze the polymerization of acrylamide in preparing polyacrylamide gels for protein separation. [7]

Tris
Tris(hydroxymethyl)aminomethane**,** $(HOCH_2)_3CNH_2$, a common component in pH buffering solutions due to its low cost and wide applicability. Tris has an effective buffering range between pH 7.1 and 9.0 which coincides with the physiological buffer of most living organisms. [9]

1. INTRODUCTION

1.1 Significance

Interferon gamma (IFNγ) is an immunomodulatory cytokine hormone of the interferon family. It has a major role in coordinating expression of immunologically relevant genes overall the human body. The cellular effects of IFNγ activity predominantly influence macrophage function in inflammation and acquired immunity during infection. [16]

Macrophages are cells with various functions in inducing an effective immune response to invasive pathogens. After its discovery, interferon gamma was at first called the macrophage-activating factor. It has been discovered later that IFNγ also coordinates other aspects of both innate and acquired immune responses, such as leukocyte attraction to the site of infection, enhancing natural killer cell activity and regulating immunoglobulin production. [17, 18]

IFNγ acts via binding to the extracellular domains of its two specific transmembrane receptors, IFNγR1 and IFNγR2, which consequently bind signaling proteins on their intracellular domains. Mutations in IFNγ and its receptors, as well as both over- and under-production of IFNγ, have been associated with several types of diseases. Mice with dominant negative mutations of IFNγ and IFNγR1 have shown deficiencies in resistance to various bacterial, viral and parasitic infections. [19] Human patients with detected inactivating mutations in IFNγ receptors have displayed susceptibility to mycobacterial and tuberculosis infections leading to death at childhood. [20]

Interestingly, some natural IFNγ polymorphisms have been connected to increased longevity. The hypothesis states that, while moderate polymorphism of IFNγ does not significantly defect an individual's ability to clear infection, it decreases the probability of exaggerated inflammatory responses, thus preventing inflammation related diseases such as asthma, osteoporosis, cardiovascular disease and neurodegeneration. [21] Increased IFNγ production has been linked to autoimmune pathology, suggesting contribution to development of e.g. diabetes mellitus and multiple sclerosis, while IFNγ-defect mice have been shown to display compromised tumor rejection. [22]

The various diseases that IFNγ has been associated with make it an appealing target for drug development. While the variety of its immunological functions underline the importance of IFNγ-targeted drug design, its multiple roles also make drug development challenging. Without detailed knowledge about the molecular mechanisms of IFNγ binding to its membrane receptors and the consequent intracellular signaling, drug targeting to effect specific functions is not possible.

Antibodies against IFNγ have been designed with pharmacological purposes, and many are currently under development. However, the comprehensive study of IFNγ, its receptors, related signaling proteins and putative antibodies, requires more efficient methods to express and purify these proteins than are available today.

1.2 Problem statement and thesis contributions

The purpose of the described study was to first express and purify functional IFNγ and its signaling complex components, IFNγR1, IFNγR2 and related JAK-1 signaling protein. After confirming functionality of the complex, the binding mechanism of putative IFNγ antibodies to the signaling complex would be examined.

The immunological role of interferon gamma has been studied extensively within the past two decades and the three dimensional structure of IFNγ alone was determined with x-ray crystallography by Ealick et al. already in 1991. [23, 24] The study of IFNγ in relation to its receptors and the signaling mechanism, on the other hand, is still preliminary. Only the extracellular domains of the two receptors have been examined, while the allosteric mechanism of receptor function remains undiscovered. [25-29]

Constructing a stable and functional signaling complex in vitro would enable observing conformational changes in the transmembrane and intracellular domains of the receptors upon IFNγ binding. This is required to ultimately understand the allosteric mechanism of how ligand binding in the extracellular domain of a transmembrane receptor leads to the binding of signaling proteins in the intracellular domain of the complex.

In order to determine in detail the signaling mechanism of the IFNγ complex through the cell membrane, the two receptors need to be expressed and purified in full length, including the transmembrane and intracellular domains. The extraction, solubilization and purification of transmembrane proteins in vitro is a far more challenging task than that of proteins which are found natively in soluble state.

Some transmembrane proteins have been successfully expressed and studied, most notably rhodopsin, the photoreceptor of retinal rod cells. In this study the protocols for expression and purification of rhodopsin were applied and modified in order to obtain functional IFNγ and its two receptors in full length.

The structure of expressed and characterized proteins of IFNγ receptor complex was analyzed with nuclear magnetic resonance spectroscopy (NMR). Finally, the binding of putative antibodies against IFNγ was assessed and the conformational changes in IFNγ upon antibody binding were examined using NMR spectroscopy.

2. BIOCHEMICAL BACKGROUND

2.1 The central dogma: DNA, RNA and proteins

A cell is considered to be the fundamental unit of all living matter. A cell is an entity separated from other cells by the cell membrane, or plasma membrane, through which it interacts with other cells. The contents enclosed by the cell membrane constitute the cytoplasm. All cells contain membranous structures called organelles and complex chemical components, which are collectively called macromolecules: nucleic acids, proteins, polysaccharides and lipids. [30]

A cell is also a device for storing and managing information. The genetic code of an individual is stored in the nucleus of each of its cells, in the sequence of nucleotides of deoxyribonucleic acid molecules. There are two types of nucleic acid molecules in the cell, deoxyribonucleic acids (DNA) and ribonucleic acids (RNA). Nucleic acids are long linear polymers composed of five different types of nucleotides: adenosine, cytosine, guanine, thymine and uracil. [30]

In the cell nucleus DNA usually forms a nucleoprotein, a DNA-protein complex called a chromosome. Eukaryotic cells usually contain several chromosomes, each cell of the same organism containing the same number of chromosomes. A *gene* is defined as a segment of DNA that encodes the sequence information of a specific protein, while *genome* is the entirety of genes encoding a whole living organism. A triplet of nucleotides in DNA representing a specific amino acid is called a codon. The sequence of codons in DNA determines the sequence of amino acids in the protein. [30]

Like DNA, RNA is a polymer of nucleotides, but whereas DNA consists of two polymer chains entwined together in a double helix, RNA is single stranded. In most living organisms, DNA contains the genetic code of the organism, but in RNA-viruses hereditary information is encoded in RNA. In all kinds of organisms, RNA has a role in transmitting the genetic information from the nucleus to the rest of the cell and translating it to form proteins. [31]

Messenger RNA (mRNA) carries the information of the genetic DNA sequence out of the nucleus. The formation of an mRNA molecule as a complementary copy of the DNA sequence is called *transcription*. The process of linking amino acids together to make proteins, called *translation*, is conducted by transfer RNA (tRNA). Translation takes place in the surface of ribosomes, which are structures composed of proteins and ribosomal RNA (rRNA). [31]

Proteins are macromolecules composed of linear polymers called polypeptides, which are amino acid chains linked by peptide bonds. There are 21 common amino acids

found in cells out of which 20 are involved in constructing human proteins. Proteins are the major functional molecules of life with an enormous number of different biochemical properties and structures. [32]

The central dogma refers to the concept of information flow from genetic encoding to constructing a whole living organism. Genomic information is mediated from DNA to RNA via transcription, from RNA to proteins via translation and transmitted from generation to the next via replication of the genome in the cell nucleus. [33]

2.2 Amino acids

Amino acids mostly consist of carbon, hydrogen, nitrogen and oxygen, although two of them, cysteine and methionine, also contain sulfur. Selenium containing selenocysteine is not present in human proteins but can be found for example in *Escherichia coli* bacteria. All amino acids contain a carboxylic group (-COOH), an amino group ($-NH_2$) and a varying side chain (-R), which determines the specific chemical properties of the type of amino acid. Amino acids showing similar chemical properties are grouped into amino acid families. Nonpolar aliphatic amino acids contain a linear side chain, whereas nonpolar aromatic amino acids contain a phenyl ring in the side chain. Polar amino acids can be uncharged, negatively charged or positively charged. [33]

Except for the simplest one, glycine, amino acids are all chiral molecules with a tetrahedral α-carbon at the center. Proteins consist solely of the L-form of each amino acid, rotating the plane of plane-polarized light counterclockwise. A schematic representation of an amino acid in its L-form is displayed in Figure 2.1. Stereospecificity is an essential property of proteins and a characteristic feature of biomolecular interactions. [33]

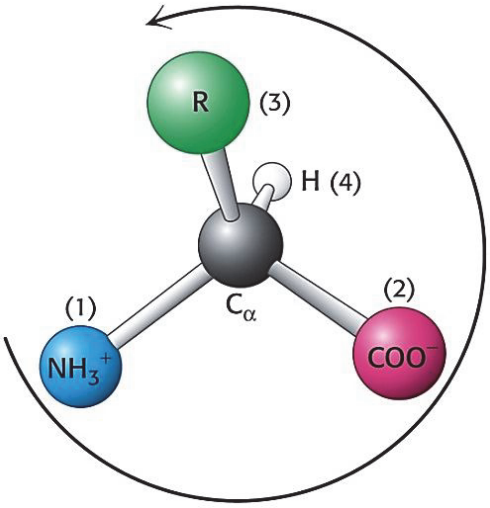

Figure 2.1 *A chiral amino acid in its L-form. Modified from Campbell.* [33]

In a neutral solution amino acids exist as zwitterions containing both negatively charged ($-COO^-$) and positively charged ($-NH_3^+$) functional groups. In a polypeptide a covalent peptide bond (-CO-NH-) forms between the α-amino-group of one amino acid and the α-carboxyl group of another one. The peptide bond has a partial double bond nature, as can be seen in Figure 2.2. This causes the planar structure of the polypeptide chain backbone, called amide plane. [31]

Short polypeptide chains consisting of up to 20 amino acids are called oligopeptides, while proteins can consist of anything from 50 to thousands of amino acids. The amino acid sequence of the polypeptide chain is referred to as the *primary structure* of the protein. According to commonly agreed notation, every protein starts with an $-NH_2$-group at the N-terminal and ends with a -COOH-group at the C-terminal. [32]

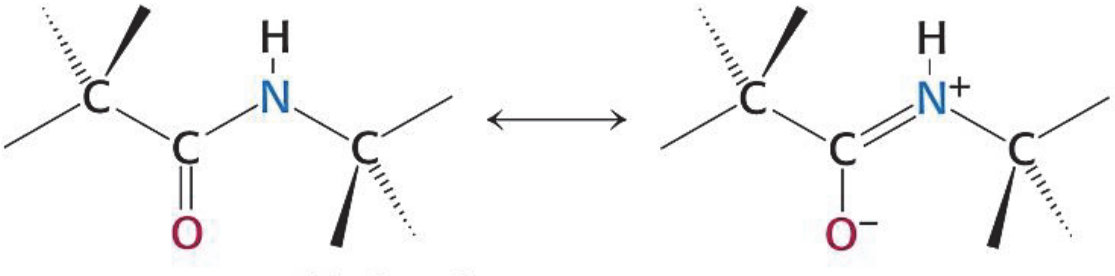

Figure 2.2 *The resonance structure of a peptide bond. The charge distribution causes the partial double bond nature of the bond.*

2.3 Secondary and tertiary structure of proteins

Despite the planar structure of the backbone, proteins do not exist in nature as elongated polypeptide chains. The most common *secondary structure* found in proteins is a right-handed helical coil called α-helix. The helical turn is held in place by hydrogen bonds forming between a hydrogen atom of a peptide bond and an electronegative oxygen of another peptide bond further in the chain. The α-helix is a highly regular structure in which each 360° turn incorporates 3,6 amino acid residues and is 0,54 nm long. While also left-handed α-helices exist, the right-handed turn is the more frequently found direction. [34]

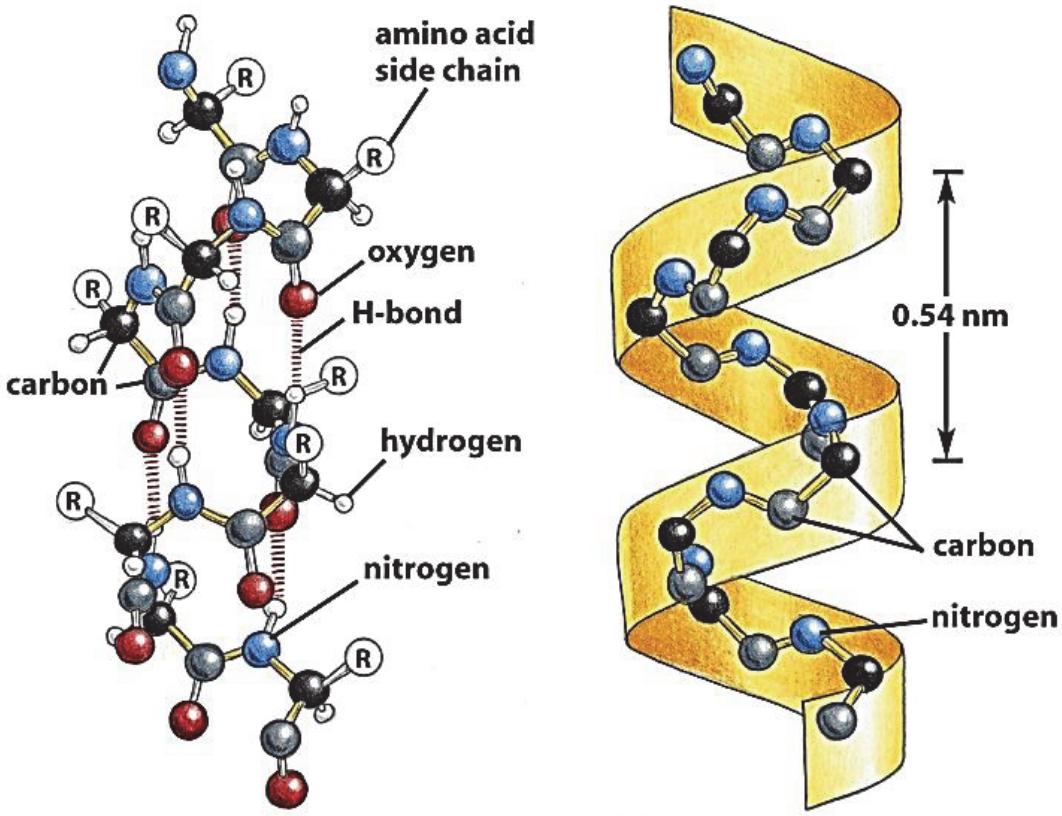

Figure 2.3 *The structure of an α-helix. The hydrogen bonds holding the structure in place are presented as red dashed lines on the left side ball-and-stick representation.* [35]

Another common secondary structure is the β-sheet in which the polypeptide chain folds back and forth upon itself, and allows intramolecular hydrogen bonding to take place. Also the β-sheet has a determined structure with one bend being 0,7 nm in length. Other existing secondary structures are β-bends, 3_{10}-helices, polyproline helices and collagen triple helices. [33]

The type of folding is determined by the available opportunities for hydrogen bonding and hydrophobic interactions, which are dictated by the amino acid sequence of the polypeptide chain. Many polypeptides fold into two or more segments with different secondary structures. These segments have specific functions in the final protein and they are commonly referred to as protein domains. [34]

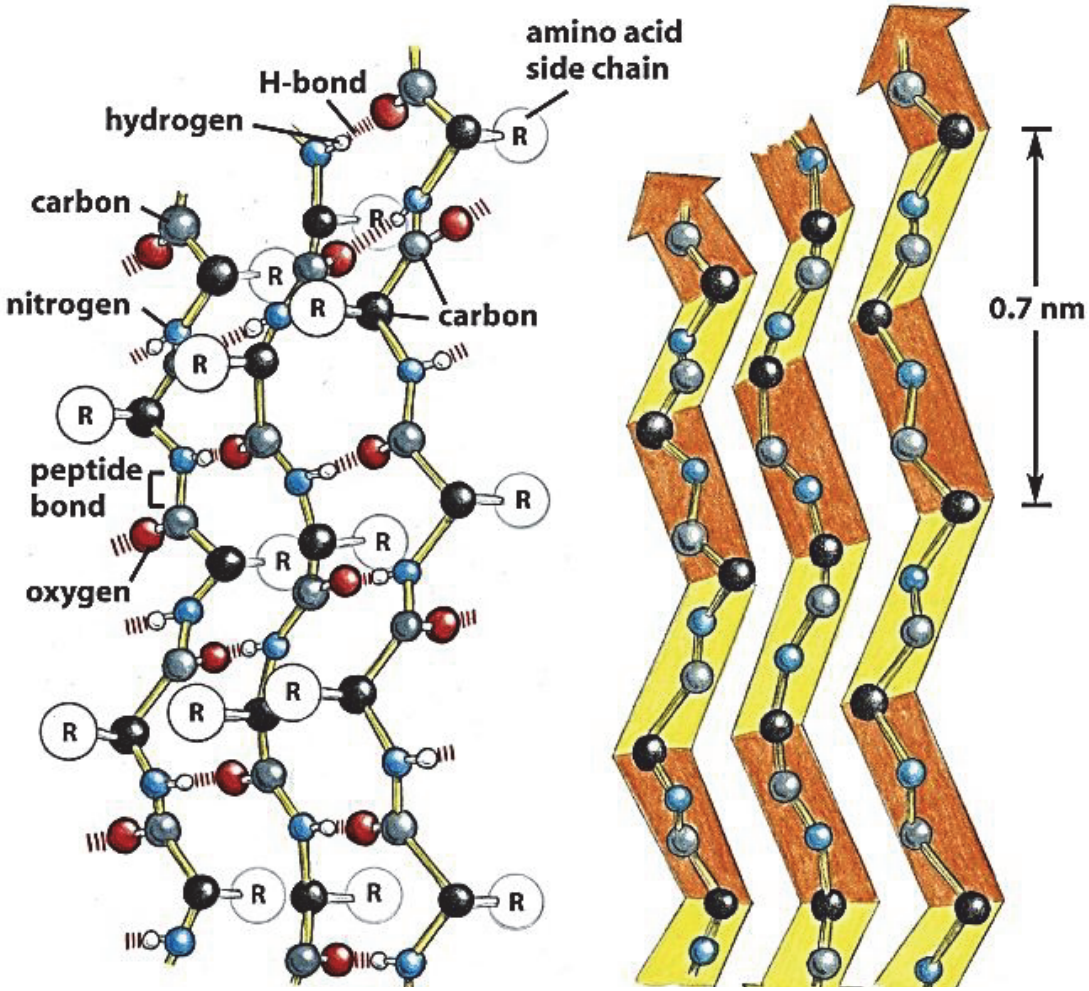

Figure 2.4 *The structure of a β-sheet. If the amino-terminal to carboxyl-terminal orientations of adjacent chains are the same a parallel β-sheet forms. If the orientations of the chains are opposite an anti-parallel β-sheet is formed.* [35]

The secondary structure of the polypeptide positions amino acids in a way that enables other intramolecular interactions, such as forming of covalent bonds, disulfide bonds, hydrophobic interactions, van der Waals forces or additional hydrogen bonds. The secondary structure thus folds back upon itself and constructs the *tertiary structure* of the protein. [32]

Often proteins consist of more than one polypeptide. The arrangement of the polypeptide subunits and the interactions holding them together are referred to as the *quaternary structure* of the protein. Some proteins exhibiting quaternary structure consist of several identical subunits, whereas others may contain many different kinds of subunits. For example, a protein consisting of three identical subunits is called homotrimeric, while another one consisting of two different subunits is heterodimeric. [32]

2.4 The structure-function paradigm

The distinctive structures of proteins allow them to conduct an enormous variety of functions in the cell. Proteins form structural entities, mediate both intracellular and intercellular signaling, transport small molecules and catalyze metabolic reactions. The entire complement of proteins expressed by the genome of an organism is termed *proteome*. [32]

The structure of a protein determines the ways in which it can interact with other molecules, be it other proteins, nucleic acids, oligopeptides, ions or small molecules. Consequently, it is the interaction with other molecules that defines the biochemical function of a protein. This is called the *structure-function paradigm* of molecular proteomics, in which it is assumed that the function of a given protein can be predicted by its structure or vice versa. [34]

Proteins are classified into families and superfamilies based on similarities in their structure. It is often found that proteins, which are unquestionably considered members of the same family, based on their nearly superimposable quaternary, tertiary and secondary structures only share down to 20% of the same amino acid sequence. This is explained by the fact that not all amino acids in a protein interact with other molecules. It is only defined residues of a protein, which participate in these interactions, at locations called binding sites or functional sites. [36]

In the traditional structural biology approach, the function of a protein is known first and only if it is considered sufficiently important would predicting the protein's structure be of interest. In an alternative approach, the structure of a protein of interest is determined based on its sequence and possible protein folds experimentally and functional sites are predicted based on the structure. [37]

Only a small number of residues would be expected to be highly conserved through a protein family. These residues are usually situated at the binding sites and are thus essential for maintaining a common function of the family. In protein science, identification and characterization of the binding sites is crucial for unveiling the recognition mechanisms which enable interaction of a protein with specific molecular partners and consequently for understanding the function of a protein. [36]

There are three levels at which protein function can be defined. At cellular level, the function of a protein involves its location in the cell and interaction with other molecules in its location. A protein's physiological function refers to the metabolic pathway in which it is involved, and the phenotypic function encompasses the role of the protein in the whole organism. [37]

It is worthwhile keeping in mind that proteins tend to bare multiple functions in an organism, or even in a single cell, and proteins can gain and lose functions during evolution. This means that proteins exhibiting a single fold can perform diverse biological functions and conversely that a single function can be achieved by many variable protein folds. [37]

2.5 Allostery in proteins

Allostery has been defined as a process in which remote sites of a system are energetically coupled to produce a functional response. Models have been created to explain how allostery works in multimeric proteins but the detailed mechanisms at the atomic level have not being unveiled to date. [38]

In proteins, allostery is understood as the phenomenon in which a stimulus at one site of a protein causes a response at another functional site of the protein. The functioning of a membrane receptor protein is by definition allosteric as they induce a response on one side of the cell membrane (effector site) to a signal received on the other side (receptor site). Allosteric regulation takes place by shifting the equilibrium between distinct functional states, which correspond to certain conformational states. [39]

Even though the amino acid sequence of a protein is constant, it's three dimensional structure is by no means static. To conduct their multiple functions, proteins dynamically change conformation. Instead of a single form, the structure of a protein is actually equilibrium of an ensemble of conformations. [38]

Despite the lack of knowledge about the specific mechanisms of allostery in proteins, it has been a common hypothesis that an allosteric reaction pathway is essentially a series of discrete conformational distortions within the protein. This notion is challenged when the structure of a protein is viewed as a continuously fluctuating ensemble of conformations. It has been suggested instead, that an allosteric pathway is an energetic linkage between remote functional sites caused by one Boltzmann distribution of states transitioning to another. [21, 40]

This spontaneous fluctuation in protein structure has been described as transitions from a folded state, or ground state, to excited states around the folded structure. The changes in conformation result from variation in local energy levels. This energetic variation may be caused by catalytic activity, binding or ambient thermal motion. Regardless of the source of energy, its effect on protein conformation depends on both enthalpic factors like thermodynamic protein-solvent interactions and entropic factors such as the degree of organization of the protein-solvent system. [38]

Wrabl et al. recently hypothesized that as spontaneous conformational fluctuations are tightly linked to modulation of protein function, functional sites might be directly coupled to these fluctuations. This would mean that functional sites of a given protein could be identified by measuring how energetic perturbations affect its conformational variation. [38]

Liu et al. analyzed for a diverse set of proteins the so-called global cooperative response (GCR), which quantified the energetic effect of a point mutation on the cooperativity between all other residue pairs in the protein. Statistically significantly higher values for GCR were identified for energetic perturbations at functional sites of studied proteins. This suggests that binding sites exhibit a high intrinsic ability to affect the energetic coupling throughout a protein, in addition to the known binding mechanisms due to chemical and structural complementarity of the binding site and its ligand. [41]

At the moment, the most advanced methods for determining protein structure are x-ray crystallography and high resolution electron microscopy which give us a detailed picture of the ground state of the protein in question but fail to give insight to its excited conformational states. Computational methods are constantly developed to model the energetic and conformational diversity of a protein ensemble. [38]

2.6 Membrane and soluble proteins

In a living organism, proteins can be found either as forming parts of cell and organelle structures or “floating” in intra- and extracellular fluids like the cytosol and blood. The structural proteins are membrane proteins and the latter are called soluble proteins. The polarity and hydrophobic forces of the environment affect the composition of the protein. In soluble proteins charged and polar residues are arranged on the water-accessible surface. In membrane proteins on the other hand, the lipid-exposed surface is covered with hydrophobic residues. [42]

Membrane proteins are usually classified in three types: intergral, peripheral and lipid-anchored membrane proteins. Integral membrane proteins are firmly linked to the membrane and are commonly found penetrating the lipid bilayer of a membrane from the extracellular side to the intracellular side and are thus called transmembrane proteins. It has been estimated that integral membrane proteins constitute 20-30% of all human proteins. Peripheral membrane proteins are located outside the membrane but are temporarily attached to it by non-covalent interactions such as electrostatic interactions and hydrogen bonding. Lipid-anchored proteins are also situated outside the membrane but are covalently attached to lipid molecules of the membrane lipid-bilayer. [43]

A different but also quite common classification is to divide membrane proteins to integral and amphitropic membrane proteins. The definition of amphitropic membrane proteins is that they can be found both connected to a membrane and soluble in the cytosol or within the extracellular matrix. They have a covalent (lipid-anchored) or non-covalent interaction with the membrane, which is reversible and usually regulated by for instance ligand binding or phosphorylation. [44]

2.7 The G-protein coupled membrane receptors

2.7.1 Function and classes of G-protein coupled receptors

G-proteins are GTPases, hydrolase enzymes which bind and hydrolase guanosine triphosphate (GTP). G-proteins form a large superfamily of proteins with a highly conserved G-domain in which the binding and hydrolysis of GTP takes place. By function G-proteins are molecular binary switches; the active state of a G-protein-GTP-target complex is switched off by hydrolysis of GTP to GDP, which causes the inactivation and release of the G-protein from its target. The G-protein is then recycled and activated again by phosphorylation of GDP to GTP. [32]

Members of the G-protein family have a critical role in a number of different cellular processes; signaling for cell division, growth and differentiation, synthesis and intracellular movements of proteins and sensory perception. Defect in G-proteins lead to a variety of diseases, including at least 25% of cancers involving a mutation in Ras-family of G-proteins. [34]

Membrane receptors that are specific to G-proteins are called G-protein coupled receptors (GPCR). The same protein superfamily might also be referred to as G-protein linked receptors (GPLR), seven-transmembrane domain receptors, or 7TM receptors or heptahelical receptors. GPCRs are cell membrane receptors, which consist of seven transmembrane helices and a cytoplasmic eight helix running parallel to the cell membrane. [45]

GPCRs function in mediating signaling transduction cascades by activating G-proteins in the cytosol as a response to extracellular ligand binding. The active cytosolic receptor site binds a cognate heterotrimeric G-protein ($G\alpha\beta\gamma$) and enables the catalysis of GDP $\rightarrow$ GTP in its $G\alpha$-subunit. GPCR ligands include various types of hormones, neurotransmitters and other small molecules and peptides. [45]

Nearly 800 human genes have been predicted by sequence analysis to code for different GPCRs. The superfamily has been divided into classes by several different schemes but the most common one is to distinguish between three main families: A. Rhodopsin-like receptors, B. Secretin receptors and C. Metabotropic glutamate receptors. Between the

main classes, no sequence homology has been detected, even though they still share the same heptahelical structure and the same signal transduction mechanism. [45]

2.7.2 Rhodopsin as a model system

The largest of the GPCR families is the Rhodopsin-like receptor family, which is divided into 16 classes depending on the type of ligand the receptor binds. There are approximately 670 human genes encoding a protein of the Rhodopsin-like family. [46]

Rhodopsin is the photoreceptor of retinal rod cells. It was the first GPCR with a defined tertiary structure and it's considered the prototypical G-protein coupled receptor due to the large amount of experimental information available concerning both its structure and function. The structure of rhodopsin is used as a model for structure prediction of newly discovered or uninvestigated GPCRs. The activation of rhodopsin happens by photoisomerization of its ligand from 11-*cis*-retinal to all-*trans*-retinal. The absorption of a photon causing the *cis* $\rightarrow$ *trans* isomerization takes place in the receptor-bound ligand *in situ*. [45]

The studied GPCRs tend to share a conserved structural core with more differences in the ligand binding regions. Since the activation of rhodopsin is conducted via isomerization of a covalently bound hydrophobic ligand the extracellular domain shielding it is very compact, compared to other ligand binding activated GPCRs with more flexible extracellular domains. Smaller variations in the cytosolic transmembrane connecting loops are responsible for the selectivity of GPCRs towards different G-proteins. [45]

The general mechanism of rhodopsin-mediated signaling is a stepwise sequence of conformational changes from local to global scale. The retinal ligand undergoes a local conformational change, which is communicated to a global conformational change throughout the protein. This global change then results in the activation of a G-protein and consequent signal amplification. This type of coupling of local and global changes within a protein might be the general scheme of allosteric signal transduction in membrane protein. [47]

2.8 Enzyme linked receptors

2.8.1 Function and classes of enzyme linked receptors

Enzyme linked receptors are also known as catalytic receptors since they mediate cell signaling by triggering enzymatic reactions in the cytosol. It is a unique feature of

enzyme linked receptors that their cytosolic domain carries intrinsic enzymatic activity. [48]

All enzyme linked receptors are single-pass transmembrane receptors, meaning that they consist of only one chain spanning the membrane from the extracellular to the intracellular side. Enzyme linked receptors are classified into five classes depending on the types of enzymes they activate: A. Guanylate cyclase receptors, B. Tyrosine phosphatase receptors, C. Serine/threonine kinase receptors, D. Tyrosine kinase associated receptors and E. Tyrosine kinase receptors. [48]

The large superfamily of tyrosine kinase associated receptors includes several immunomodulatory receptor families such as cytokine receptors, antigen specific receptors on lymphocytes, interleukin receptors, erythropoietin receptors and also growth hormone receptors. When a ligand binds to a tyrosine kinase associated receptor, the associated tyrosine kinase catalyzes the covalent phosphorylation of tyrosine within specific intracellular signaling proteins in order to initiate or increase their signaling activity. [48]

Allosteric regulation is similar in GPCRs and enzyme linked receptors in the sense that they act via coupling to a separate transducer protein in the cytosol. Ion channels on the other hand only conduct conformational changes within the receptor itself. [39]

2.8.2 Interferon gamma receptor signaling complex

Interferon gamma, also written interferon-γ or IFNγ, is a homodimeric inflammatory cytokine, which mediates immunologic signaling by via two cytokine receptors, IFNγR1 and IFNγR2. Hormones in the cytokine superfamily act through a mechanism of hormone-inducing receptor aggregation. Interferons including IFNα, IFNβ and IFNγ participate in antitumor and immunomodulatory signaling and in producing a host defense response to viral and bacterial infections. IFNγ is predominantly produced by activated T-cells and natural killer cells throughout the body. [19]

Interferons are divided into two distinct types based on the structure of their signaling complexes. IFNα and IFNβ are type I interferons displaying a common four-helix bundle structure and cross-reactivity with shared receptors. IFNγ is the only type II interferon. It shows a high degree of receptor specificity as its two receptors only respond to IFNγ. In its biologically active from the structure of IFNγ is a symmetric homodimer with an unusual pattern of interdigitating α-helices. [29, 49]

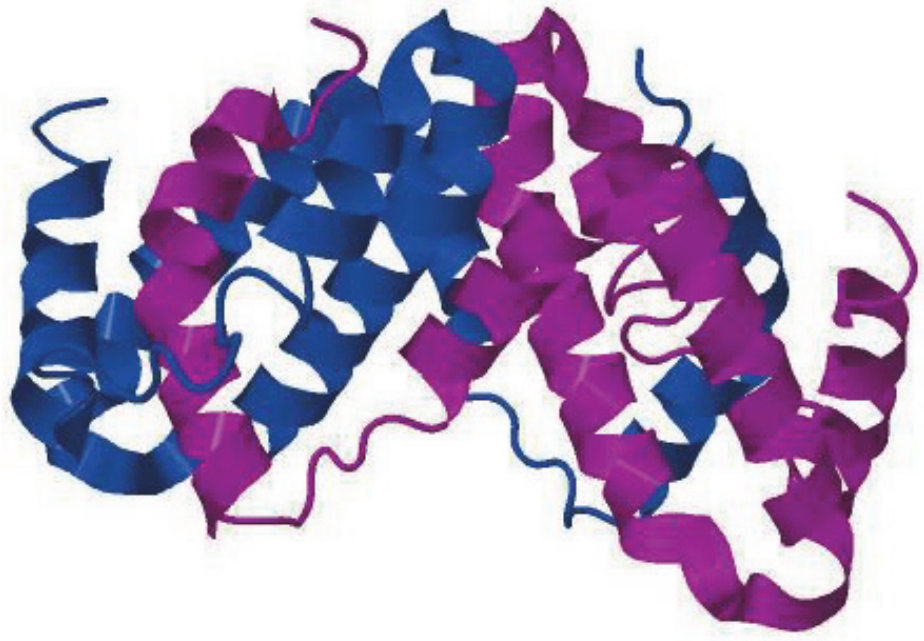

Figure 2.5 *The structure of an IFNγ molecule. The two interdigitating monomers are displayed in distinct colors.*

IFNγ mediates by binding to the extracellular domains of its two receptors. IFNγR1 and IFNγR2 are both type II single transmembrane receptors and members of the cytokine receptor family. They differ in their affinity toward the ligand, IFNγR1 being a high affinity receptor while IFNγR2 is a low affinity receptor. The IFNγ homodimer is bivalent and has been found to bind symmetrically two copies of each receptor in a 1:2:2 stoichiometry. [27, 48, 50]

Due to the difference in receptor affinities binding takes place sequentially in two steps. A stable intermediate of IFNγ with the two IFNγR1 receptors is formed first, followed by the binding of IFNγR2 to the intermediate, possibly binding to sites both at the primary receptor the ligand directly. [51]

The detailed sequence of the binding activities is not proven up to day. It has been suggested previously that formation of a functional complex with only one copy of each receptor is also possible with a 1:1:1 stoichiometry, although it only retained about 1% of wildtype activity. On the other hand, the crystallographic structure observed by Thiel et al. gave a surprising result by showing an unexpected third IFNγR1 receptor bound to the IFNγ dimer. [26, 28]

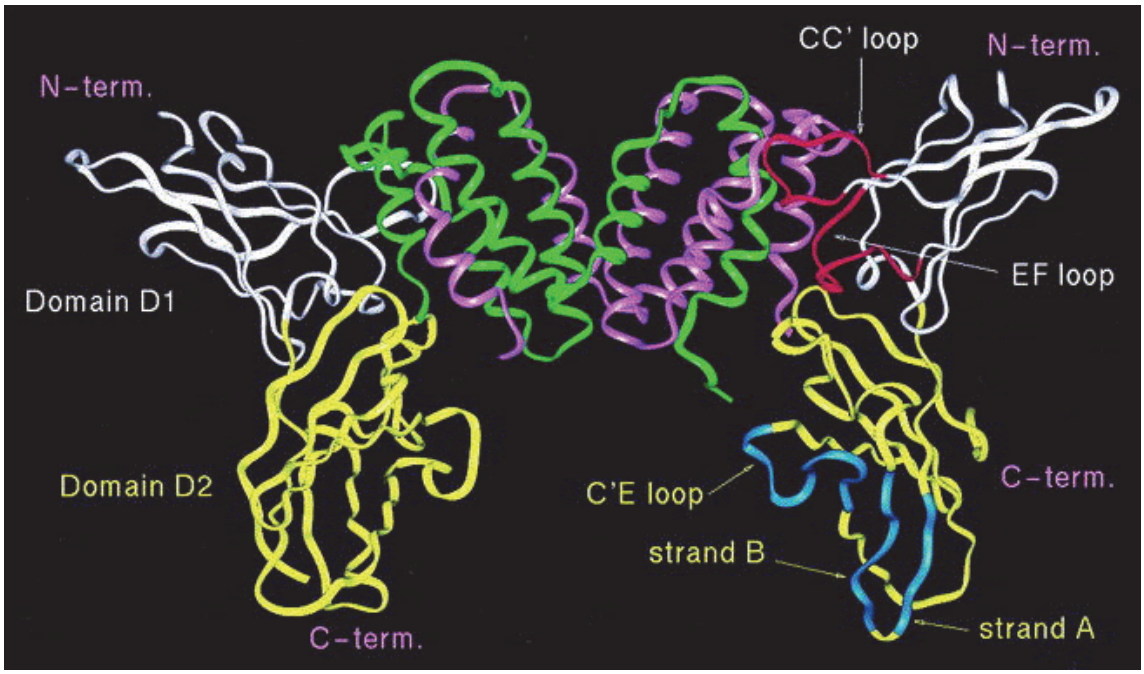

Figure 2.6 *A ribbon representation of a 1:2 IFNγ : IFNγR1 intermediate complex (with extracellular domains of the receptor), determined by Du et al. IFNγ dimer between the two receptors is pictured in purple and green.* [29]

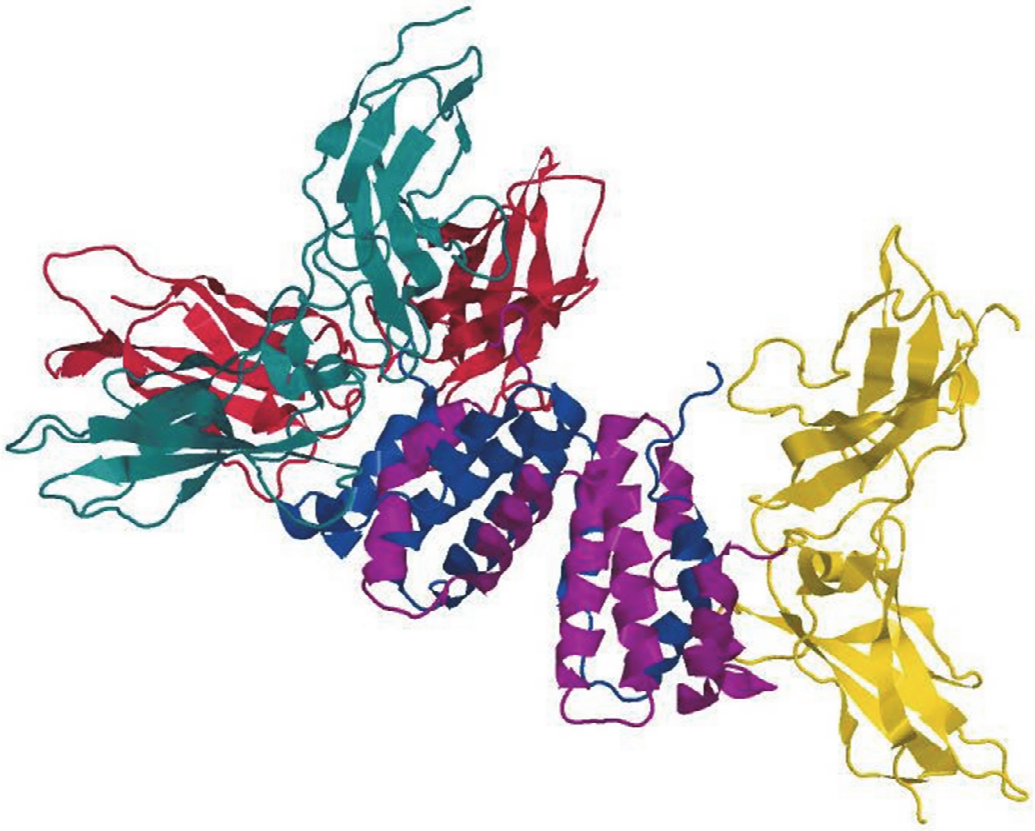

Figure 2.7 *The structure of an IFNγ signaling complex with unexpected three IFNγR1 receptors (red, teal and yellow) bound to the IFNγ dimer (blue/purple).* [51]

The activation of IFNγR1 and IFNγR2 initiates a signaling cascade in which Janus tyrosine kinases (also called Janus kinases) bind to IFNγ receptors, Jak-1 to IFNγR1 and Jak-2 to IFNγR2. Janus kinases regulate the phosphorylation of STAT-1 (Signal Transducer and Activator of Transciption) and its translocation to the nucleus of the cell in order to enable the initiation of a transcription process. [52]

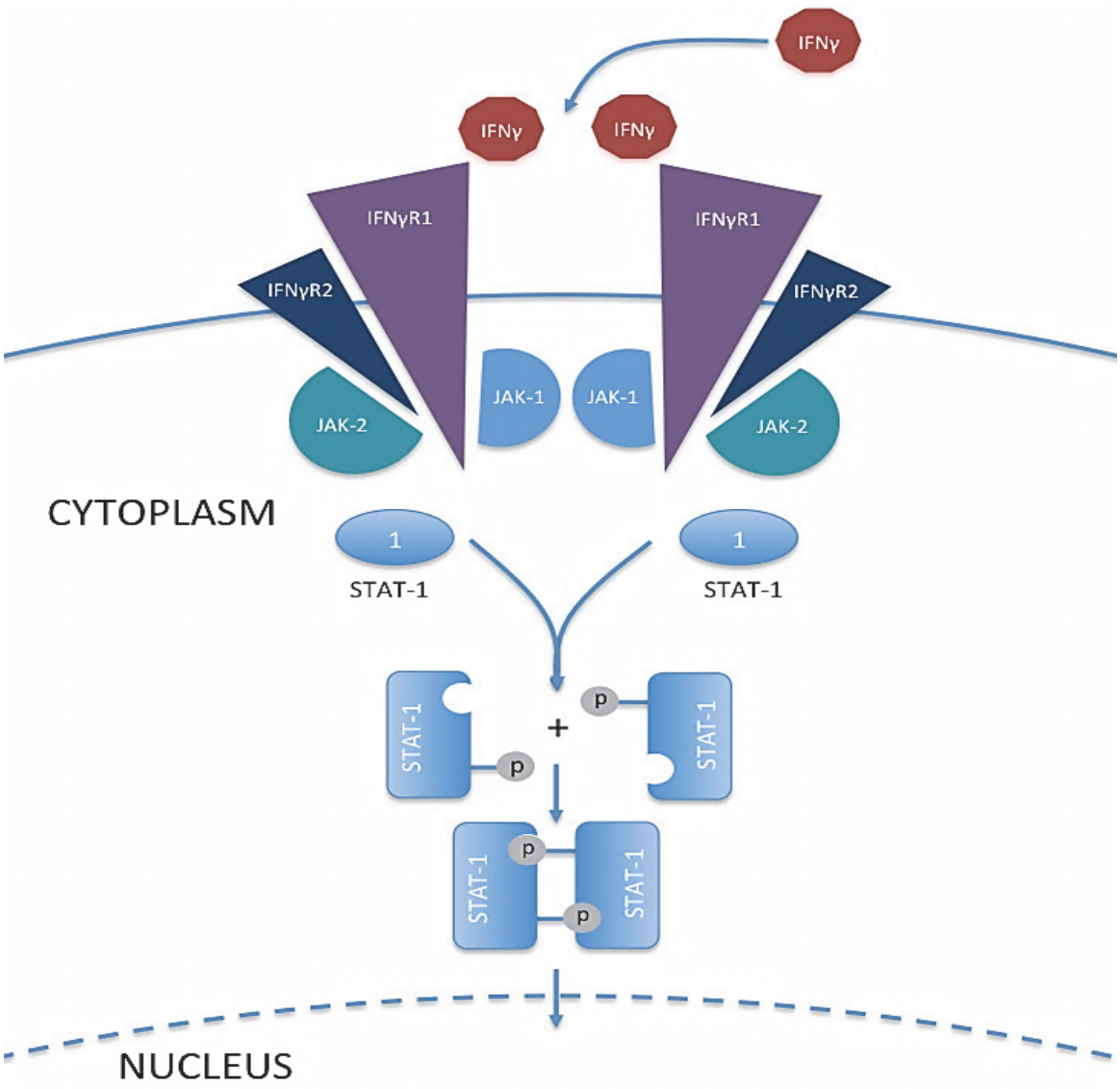

Figure 2.8 *A schematic representation of the IFNγ signaling pathway. The binding of the IFNγ to the extracellular domain of IFNγR1 initiates a signaling cascade in which Jak-1 and Jak-2 activate phosphorylation of STAT-1 and STAT-2 enabling them to pass through the nuclear envelope.*

2.9 Ion channels

Due to unequal distribution of ions in the cytoplasm and the extracellular matrix, there is a membrane potential across the plasma membrane of each cell. Changes in membrane potentials are the basis for signal transduction between adjacent nerve cells. At the synapse between two neurons, the depolarization of the pre-synaptic cell membrane releases a flow of neurotransmitters, which in turn depolarizes or hyperpolarizes the membrane of the post-synaptic cell. [39]

Changes in membrane potentials are caused by ion currents across the membrane. Ion channels are specialized transmembrane proteins, which carry the ion currents against the energy barrier of the polarized membrane. Ion channels are highly selective to their target ions and they are activated by specific signals such as neurotransmitters or other ligands. [39, 53]

All ion channels are composed of two functional domains: a selectivity filter to sort only certain types of ions to pass the channel, and a gate to determine when the specified ions are allowed to flow through the channel. Ion channels are divided into voltage-gated ion channels (VGIC) and ligand-gated ion channels (LGIC) depending on whether their activation is triggered by a change in membrane potential or by attachment of a molecular ligand. [54]

3. RESEARCH MATERIALS AND METHODS

3.1 Molecular biology

3.1.1 Plasmids

The recombinant DNA for human IFNγ, IFNγR1, IFNγR2 and caspase-1 were coded in two different mammalian expression vectors: pMT4 for transient transfection and pACMV for stable transfection. The plasmids were prepared in Institute for Structural Biology, Research Center Jülich in Germany, as collaboration with assistant professor Judith Klein-Seetharaman, University of Pittsburgh. A Strep-tag tag was encoded in the plasmids for IFNγ and its two receptors in order to enable high-affinity purification of the expressed proteins.

PMT4 is a member of protein O-mannosyltransferase family, which acts as an important protein modification promoter by initiating the assembly of O-mannonyl glycans. Protein O-mannosylation is an evolutionarily conserved protein modification of fundamental importance in many eukaryotes, transferring mannose residues from dolichyl phosphate-D-mannose to protein serine/threonine residues. [55]

The purpose of gene expression plasmids for mammalian cells is to promote maximum production of target mRNA. Typically mammalian expression plasmids include a strong promoter for high-level expression of the target gene, such as cytomegalovirus (CMV) promoter in pACMV. The plasmid also contains a polyadenylation sequence to stabilize the mRNA transcript, a multiple cloning site (MCS) for insertion of the target gene and a DNA sequence for antibiotic resistance to allow replication in E.coli and antibiotic selection. [56]

A Strep-tag is an oligopeptide, which is usually attached to the N- or C-terminal of the recombinant protein, or as a linker between two protein domains. The Strep-tag binds to the biotin binding pocket of streptavidin with high affinity and can be eluted with biotin derivatives. Strep-tagging is a popular method for studying recombinant protein-protein interactions as it enables a reliable and highly specific purification in variable conditions. Strep-tagged proteins can also be examined with nuclear magnetic resonance (NMR) spectroscopy, which further facilitates the study of structure and function of the purified proteins. [57]

Jak1-GST was also obtained from Institute for Structural Biology Research Center Jülich. The GST-tag is a glutathione S-transferase gene fusion system in which the GST sequence is incorporated into an expression vector with the gene encoding the protein of

interest. Protein expression results in expression of the protein of interest fused to the GST protein, which can be purified via its high affinity for glutathione. [58]

3.1.2 Transformation

In order to increase the amount of available DNA, a transformation in E.coli was conducted for the DNA of all proteins of the IFNγ-receptor complex. In total seven DNA samples were transformed:

1. Control with no DNA
2. Jak1-GST
3. IFNγR1 in pMT4
4. IFNγR1 in pACMV
5. IFNγ in pMTV4
6. IFNγ in pACMV
7. IFNγR2 in pMT4
8. IFNγR2 in pACMV

The transformation was conducted with the following procedure. 0,5 µl of each DNA sample was placed in a 1,5 ml tube with 35 µl of E.coli cells. The mixture was centrifuged shortly. The cells were incubated on ice for 30 minutes and then exposed to a heat shock at 37°C for 45 seconds. 1 ml of LB growth medium was added into the tubes and the cells were incubated for 1 hour in 37°C. 100 µl per plate of each of the resulting bacterial cultures was spread on plates with LB medium containing Ampicillin to stop the growth of cells with no introduced plasmid DNA. The cells were let to grow overnight at 37°C and picked 13 hours later.

3.1.3 Purification of DNA

The purification of amplified plasmid DNA was conducted with a Qiagen Maxi Plasmid Kit which contained a complete set of suspension, lysing neutralization and elution buffers. A single colony was picked from each selective plate to inoculate a starter culture in 3 ml of LB medium containing Ampicillin. The starter cultures were incubated for 8,5 hours at 37°C in a shaker at 300 rpm. 500 ml of selective LB medium was inoculated with 500 µl of each starter culture in a flask of 2 liters volume. The cells were incubated at 37°C in a shaker at 300 rpm for 14 hours. The suspension was centrifuged at 4°C in 6000 g for 15 min and the supernatant was discarded. The cell pellets were stored at -20°C until proceeding to the next step.

The quality and yield of purified DNA depend on the quality of bacterial the cell lysate. Alkaline lysis is one of the most common methods used for lysing bacterial cells for

plasmid purification. SDS in lysing buffer solubilizes the protein and phospholipid components of the cell membrane, allowing the release of the cell contents. NaOH denatures released proteins and both chromosomal and plasmid DNA. The presence of RNase A causes released cellular RNA to be digested during lysis.

Before lysis the cell pellets were thawed and resuspended completely by vortexing in 10 ml of Tris-Cl-EDTA suspension buffer containing RNase A. The cells were lysed by adding 10 ml of NaOH/SDS lysing buffer in each tube and mixing by carefully inverting the tube to avoid shearing of the genomic DNA, as the sheared fragments of the chromosomal DNA could copurify with the plasmid DNA and considerably lower the quality of the target DNA yield. The lysis reaction was allowed to proceed for 5 min at room temperature.

For precipitation of KDS and neutralization of the lysate, it was mixed with 10 ml of chilled neutralization buffer containing acidic potassium acetate, and incubated on ice for 20 min. The high salt concentration of potassium acetate causes KDS to precipitate, and cellular debris, denatured proteins and chromosomal DNA to coprecipitate in insoluble salt-detergent complexes. The circular and covalently closed plasmid DNA renatures and remains in solution. The samples were mixed again and centrifuged at 4°C for 30 min in 20 000 g, and the supernatant containing the plasmid DNA was removed. The centrifugation was repeated for 15 min and the additional supernatant was added to the previously saved one. [59]

The QIAGEN-tip 500 was equilibrated by applying 10 ml of equilibration buffer and letting it flow through the column completely by gravity flow. The supernatant containing the plasmid DNA was applied to the column. The column was washed twice with 30 ml of wash buffer. The plasmid DNA was eluted with 15 ml of elution buffer and the eluate was collected in 50 ml glass centrifuge tubes.

The DNA was precipitated from elution by adding 10,5 ml of isopropanol. The solution was mixed and centrifuged at 4°C for 30 min in 15 000 g, and the supernatant was decanted promptly but carefully. The resulting DNA pellet was washed with 5 ml or 70% ethanol and the mixture was centrifuged for 10 min in 15 000 g at 4°C. The pellet was then air-dried for 10 min and redissolved in 10 mM Tris-HCl at pH 8,5.

3.1.4 Quantification and assessment of purity of DNA

The purity of transformed DNA was assessed with spectrophotometric measurements with an ultraviolet wavelength range from 200 nm to 400 nm. Ultraviolet absorption is a rapid and noninvasive method for assessing concentration for both DNA and protein.

However, it is not very accurate, so the results should be considered estimates of the actual concentration. [60]

Table 3.1 *Measured absorption of purified DNA after first transformation.*

λ	260 nm	280 nm	260/280
JAK1-GST	0,0559	0,0552	1,013
IFNγR1 (pMT4)	0,0269	0,0246	1,093
IFNγR1 (pACMV)	0,0310	0,0290	1,069
IFNγR2 (pMT4)	0,3299	0,3280	1,006
IFNγR2 (pACMV)	0,4108	0,4138	0,993
IFNγ (pMT4)	0,2738	0,2776	0,986
IFNγ (pACMV)	0,1899	0,1923	0,988

The absorption maximum for DNA is typically at 260 nm and for proteins at 280 nm. The ratio of absorbance at 260 nm verses absorbance at 280 nm for pure DNA should be between 1,7 and 1,8. [61] The measured ratio turned out to be around 1,0 for most samples from the first transformation, which shows that a considerable amount of protein debris was left in the DNA after purification.

A second transformation was conducted for receptor proteins IFNγR1 and IFNγR2 in E.coli and the cells were harvested with the same protocols as presented before. The plasmid DNA was purified again with a Qiagen Maxi Plasmid Kit but this time directly after harvesting the cells to avoid unnecessarily freezing and rethawing the cells. The purity of the DNA was again assessed with a spectrophotometer, and its quantity was calculated from absorbance at 260 nm. Absorbance was measured from a 1:19 DNA:water dilution, which gives the concentration of DNA in mg/ml as a direct reading of absorbance at 260 nm.

Table 3.2 *Measured absorption of purified DNA after second transformation.*

λ	260 nm	280 nm	260/280	c (mg/ml)
IFNγR1	1,5767	0,9537	1,6532	1,5767
IFNγR2	1,1003	0,6990	1,5741	1,1003

This time the 260/280 nm ratio was fairly good, being 1,65 for IFNγR1 and 1,57 for IFNγR2. For a total volume of 600 µl of purified DNA for both receptors, there was an estimated amount of 950 µg of IFNγR1 and 660 µg of IFNγR2.

3.2 Protein expression and purification

3.2.1 Transient transfection of COS-1 cells

For functional expression of the target gene in mammalian cells, the DNA needs to be delivered to the nucleus of the host cell efficiently and without degradation. Transfection is defined as the delivery of naked DNA into the nucleus of the host cell. For the nucleus to intake the external DNA, host cells must be treated with transfection agents together with the plasmid vector. [56]

Transient transfection provides a relatively rapid and simple method for examination of a recombinant protein. After transfection with the target gene, cells are usually incubated for 2-3 days to allow gene expression and accumulation of the target protein. Cells are then harvested for assay or purification of the target protein.

One of the most common mammalian cell lines is the COS-1 cell line which is an immortalized cell line originally derived from African green monkey kidney. COS-1 cells are routinely used for several types of recombinant gene expression. [62] The hind-side of transient transfection is that it is only convenient for a rather small-scale (microgram) protein production. Since the COS-1 only grows as adherent monolayers, many dishes are required for large-scale production, which becomes time-consuming and laborious. [56]

COS-1 cells were transfected transiently with IFNγ, IFNγR1, IFNγR2, JAK-1, and rhodopsin DNA in pMT4 vectors, using a chemical method with DEAE-dextran and chloroquine as transfection agents. DEAE-dextran is a cationic polymer which binds the negatively charged plasmid DNA and adsorbs to the also negatively charged cell membrane which otherwise would reject the external DNA. The complex is then taken in to the host cell by endocytosis. Chloroquine acts as an inhibitor of the lysosomal degradation of the external DNA after its intake, and facilitates its entry in the host cell nucleus. As chloroquine is toxic to the cells, the exposure time to it is strictly restricted. [5]

COS-1 cells were obtained from cryostock of professor Klein-Seetharaman in University of Pittsburgh department of Structural Biology. Cells were woken up from

liquid nitrogen by slowly (1 ml / 5 min) suspending them in DMEM[1] medium and centrifuging for 10 min in 3500 rpm at room temperature to diffuse remaining DMSO form the cells. The cell pellet was then re-suspended in 20 ml of DMEM on a 15 cm dish which was incubated at 37°C until it reached approximately 80–90% confluence.

Once sufficiently confluent the cells were split to six 15 cm dishes. The old medium was aspirated carefully and the dishes were washed with 5 ml of phosphate buffered saline (PBS). The cells were detached by incubating at 37°C with 2 ml of trypsin for 2-5 min and agitating the dish gently. 8 ml of DMEM was added to in activate trypsin, and the cells were removed from the dish by pipetting several times up and down. The cells were transferred to six new plates with 20 ml of DMEM each, and incubated at 37°C for four days until reaching 80-90% confluence.

Before transfection the six plates were split again with a ratio 1:5 and incubated till 90% confluence, resulting in 30 nearly confluent plates ready for transfection. Six dishes were transfected with IFNγ, IFNγR1, IFNγR2 and Jak-1 each, whereas three dishes were transfected with caspase-1 and rhodopsin each.

Before transfection the plates were aspirated and washed twice with 8 ml of DMEM without FBS. 12,5 μg of plasmid-DNA was added to each dish in 10 ml of FBS free DMEM containing 0,25 mg/ml DEAE-dextran and 0,1 M Tris-HCl (pH 8). The dishes were incubated at 37°C for 5-6 hours.

Following incubation the solution was aspirated and 0,1 mM chloroquine in 15 ml of FBS free DMEM was added. Chloroquine was allowed to function by incubating at 37°C for 1,5-2 hours, before aspirating and washing the dishes twice with 8 ml of FBS free DMEM. Finally 20 ml of full DMEM per dish was added and the cells were incubated at 37°C until the total incubation time from the addition of DNA reached 55-60 hours.

After incubation of 55 hours from the introduction of plasmid DNA to the cells, the cells were harvested. The medium was aspirated and the dishes were washed with 10 ml of PBS. Another 5 ml of PBS was added and the cells were scraped off the dish with a plastic scraper and placed in 15 ml sample tubes. The cells were pelleted by centrifuging in 4000 rpm at 4°C for 10 min. The pellets were solubilized in 1,0 ml/dish of 1% DM in PBS containing 5 μl/ml of PMSF and benzamidine. The harvested cells were flash frozen in liquid nitrogen and stored at -20°C for further assays, or protein purification was conducted directly after harvesting.

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[1] Unless otherwise specified in text, the DMEM used contained 10% of FBS and 1% of Pen-strep.

3.2.2 Stable transfection of HEK-293 cells

3.2.2.1 Construction of a stable cell line

Stable transfection aims at integrating the target gene permanently into the chromosome of the host cell. In successful stable transfection, the expression plasmid is integrated at random positions in the host chromosome. The site of integration in the chromosome and the number of target gene copies stably transfected determine the expression level.

In stable cell line construction the plasmid DNA encoding the target protein is delivered to the host cell with the same procedure as in transient transfection. In a small fraction of transfected cells a non-homologous recombination occurs between the expression plasmid and the host chromosome, and the plasmid becomes permanently incorporated into the genome.

The cells that undergo stable transfection are selected by antibiotic resistance marker in the expression plasmid. The cell cultures are fed with antibiotic containing medium, in which the majority of cells not expressing the target gene are killed, while the successfully transfected cells form clonal colonies on the culture dish. By picking the colonies, monoclonal cell lines can be established and grown to provide biomass for protein purification. [56]

The HEK-293 cell line derived from human embryonic kidney epithelial cells was chosen for its growth and post-translational modification qualities. HEK-203 cells adapt easily to growth in suspension and adhere relatively loosely to the surface of the cell culture dishes. The cell line is commonly used for expression of complex human proteins with human glycosylation, and is a relatively reliable option for cells that tend to be difficult to express, such as receptor proteins and ion-channels. [63] The pACMV vector used for stable transfection contained an antibiotic resistance marker for neomycin (Geneticin), which allowed for selection of successfully transfected cell colonies for protein production.

HEK-293 cells were transfected with IFNγR1 and IFNγR2 plasmids using DEAE-dextran and chloroquine as transfection agents, initially with the same procedure as used in transient transfection. Instead of adding the same amount of DNA to all dishes, half were transfected with 10 µl of DNA and half with 30 µl of DNA. After transfection cells were fed with DMEM-f12 medium (consisting of half DMEM, half nutrient mixture with HEPES buffer, L-glutamine and pyridoxine hydrochloride) containing Geneticin until all non-transfected cells died and only few separate colonies of the Geneticin resistant clones remained growing on dishes. Both DMEM-f12 medium and Geneticin were products of Life Technologies Corporation Invitrogen™.

In total 16 10 cm dishes of HEK-293 cells were cultured, eight with IFNγR1 and eight with IFNγR2:

- four dishes transfected with 10 µl of DNA:
  - 2 fed with 2 mg/ml Geneticin medium
  - 2 fed with 3 mg/ml Geneticin medium
- four dishes transfected with 30 µl of DNA:
  - 2 fed with 2 mg/ml Geneticin medium
  - 2 fed with 3 mg/ml Geneticin medium

Cells transfected with IFNγR1 did not survive the Geneticin treatment, while cells transfected with IFNγR2 survived and maintained several antibiotic resistant colonies growing. The resistant colonies were each picked and expanded to corresponding wells on a 12-well plate and on a 24-well plate.

In total 56 colonies were successfully picked and expanded. Four colonies were found on one dish with 10 µl of DNA and 3 mg/ml Geneticin. Eight or more colonies were found on all other dishes. In general it could be seen that slightly more colonies were growing on 2 mg dishes than 3 mg dishes but there was no difference in dishes transfected with 10 µl DNA versus 30 µl DNA.

Table 3.3 *Number of resistant colonies growing on dishes treated with 2 or 3 mg/ml Geneticin.*

plate number	IFNγR2 DNA (µl)	Geneticin (mg/ml)	number of colonies
1	10	2	> 8
2	10	2	> 8
3	10	3	4
4	10	3	> 8
5	30	2	> 8
6	30	2	> 8
7	30	3	> 8
8	30	3	> 8

Once close to confluent, the cell cultures on 24-well plates were expanded to 6-well plates with a splitting protocol previously introduced and fed again until reaching confluence.

3.2.2.2 Establishing a tetracycline-regulated expression system

In order to acquire higher-level expression of the receptor proteins, establishing a tetracycline-inducible expression system was experimented. Previously tetracycline-

inducible systems had been successfully established to produce for instance wild type and mutant opsins in large quantities (up to 10 mg/l). [64]

Some proteins have been shown to be cytotoxic and when produced by unregulated expression, even lethal to host cells. In bacterial expression systems, a frequently used strategy for expressing membrane proteins toxic to the bacterial cells is repressing the expression of the target gene until a desired cell density has been achieved, and only activating the expression by inducing the gene by treating the cells with specific chemicals. Tetracycline-regulated gene expression in mammalian cells has been developed more recently, but has already been shown to function in diverse expression systems. [65]

The protocol for tetracycline-regulated expression of IFNγR2 was adapted from the protocol described by Reeves et al. for a tetracycline-inducible expression of opsin mutants in HEK-293 cells. They had discovered that, while tetracycline alone notably increased the expression of opsins, a significantly greater increase in protein production was gained by treating the cells with sodium butyrate in combination with tetracycline. [64]

Cells on 6-well plates were thus induced to express IFNγR2 by addition of 15 µl of tetracyclin and 15 µl sodium butyrate per well. The cells were further incubated for 12 hours, before harvesting for target protein extraction and purification.

The DMEM-f12 medium was aspirated and the cells from each well were suspended in 1,5 ml of ice cold PBS in Eppendorf tubes. The cells were pelleted by centrifuging in 5 000 rpm for 5 min at 4°C. The pellets were then solubilized in 1% DM in PBS and mixed on an end-over-end mixer at 4°C for one hour. After thorough mixing the samples were again centrifuged at 4°C in 35 000 rpm for 10 min. For the samples in which a non-solubilized cell pellet emerged in centrifuging, the pellet was resuspended in CHAPS lysis buffer containing also DTT and PMSF. The clear supernatants containing the solubilized protein were flash frozen in liquid nitrogen and stored at -20°C over night for Western blotting the following day.

All 56 samples were screened for IFNγR2 expression by western blotting with a Bio-dot microfiltration apparatus from Bio-Rad. Based on the expression strength, 18 best clones were selected for further expansion of cell cultures. The 18 best clones growing on 12-well plates were expanded first to 6-well plates, and once confluent, further to 10 cm culture dishes. While expanding, the clones were maintained growing also on 6-well plates.

The cells on 6-well plates were induced for protein expression with tetracycline and sodium butyrate treatment as previously. The cells were incubated for 12 hours, before

harvesting and solubilization with the same protocol as before. All 18 previously selected samples were screened again for IFNγR2 expression with a Bio-dot microfiltration apparatus. 10 strongly expressing clones were further selected for expansion.

The corresponding clones on 10 cm dishes were allowed to grow near confluence and expanded to two 15 cm dish each. For the five best expressing clones, half the cells on 15 cm dishes were cryogenized in liquid nitrogen for future use, and half were further expanded to 15 cm dishes with a splitting ratio of 1:5.

Once the dishes were getting close to confluence, 3/5 of the cells were used to inoculate a 2 liter spinner flask, while growth was maintained on two dishes. To inoculate a flask, the cells were suspended in 500 ml of inoculation DMEM medium containing 10% FBS, 1% Pen-strep, 50 µg/ml of pluronic acid and 50 µg/ml of heparin sulfate. Flasks were incubated on magnetic stirring platforms for five to eight days depending on the growth rate of cells. One spinner flask was also inoculated with the remaining cells of all the clones, which were not selected after the 2nd expression screening, and another spinner flask was inoculated with the still growing cells of all the clones, which were not selected after the 1st expression screening.

The cells which had been growing well and without contamination were harvested and pelleted by centrifuging in 3 000 rpm at 4°C for 10 min. The pellets were washed twice with 50 ml of PBS and centrifuged again for 10 min after each wash. The washed and quickly air-dried pellets were weighed and each pellet was redissolved in 9 ml of 1% DM in PBS with 0,1 mM PMSF and 0,1 mM benzamidine.

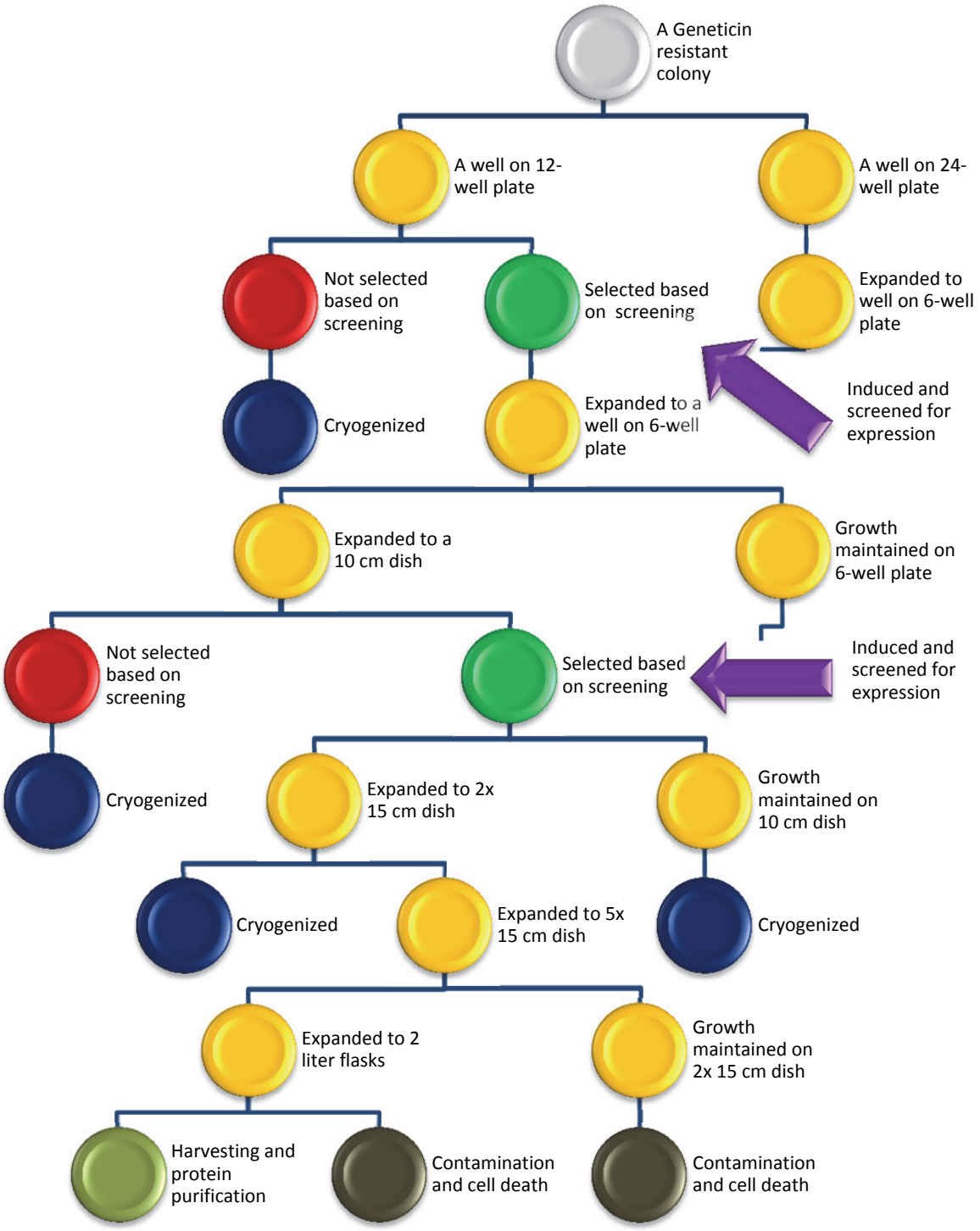

Figure 3.1 *The selection process of stably transfected IFNγR2.*

3.2.3 Purification of proteins

3.2.3.1 Affinity chromatography with 1D4 antibody

The rhodopsin produced in COS-1 cells was purified with 1D4 antibody affinity chromatography. 1D4 is an antibody that recognizes the last nine C-terminal amino acids of rhodopsin. 1D4 was coupled to Sepharose beads. [66]

The supernatant was applied through 100 µl of 1D4-Sepharose beads in a 10 ml column. The beads were washed with PBS containing 0,05% DM in order to attach rhodopsin to the antibody. The beads were washed 10 times with 2 ml of 100 mM sodium phosphate and rhodopsin was eluted with a 70 mM solution of specific C-terminal epitope nanopeptide in 100 mM sodium phosphate. Elutions were collected in five separate 500 µl fractions, with at least 10 minutes elution time for each fraction.

3.2.3.2 Affinity chromatography with Streptavidin

IFNγ, IFNγR1 and IFNγR2 were purified with Streptavidin antibody affinity chromatography. The harvested and solubilized cells were mixed with end-over-end nutator at 4°C for one hour, and non-soluble particles were removed by centrifuging at 4°C in 35 000 rpm for 30 min. 100 µl of Streptavidin beads were added per 150 µl of the supernatant. The receptor was allowed to bind to Streptavidin by end-over-end mixing at 4°C for minimum four hours, or up to 12 hours.

The protein solution was applied in a 10 ml column, and the column was washed 10 times with 2 ml of 200 mM potassium phosphate at pH 8,0 and 10 times with 2 ml of 100 mM sodium citrate at pH 8,0, both containing 0,05% of DM. The receptors were eluted in five separate 100 µl fractions of 2,5 mM desthiobiotin in100 mM sodium citrate at pH 8,0 with 0,05% DM.

3.2.4 Quantification of proteins

3.2.4.1 Absorbance spectroscopy

Purified rhodopsin control was quantified with ultraviolet absorbance spectroscopy. This quantification method is only applicable to pure protein since it is very sensitive to interfering agents. It is often used for generating protein elution profile after column chromatography in order to guide in pooling elution fractions of higher protein concentration. The absorption spectrum is based on the intense absorption around 280 nm wavelength by the aromatic rings of tryptophan, tyrosine, histidine and phenylalanine. [61]

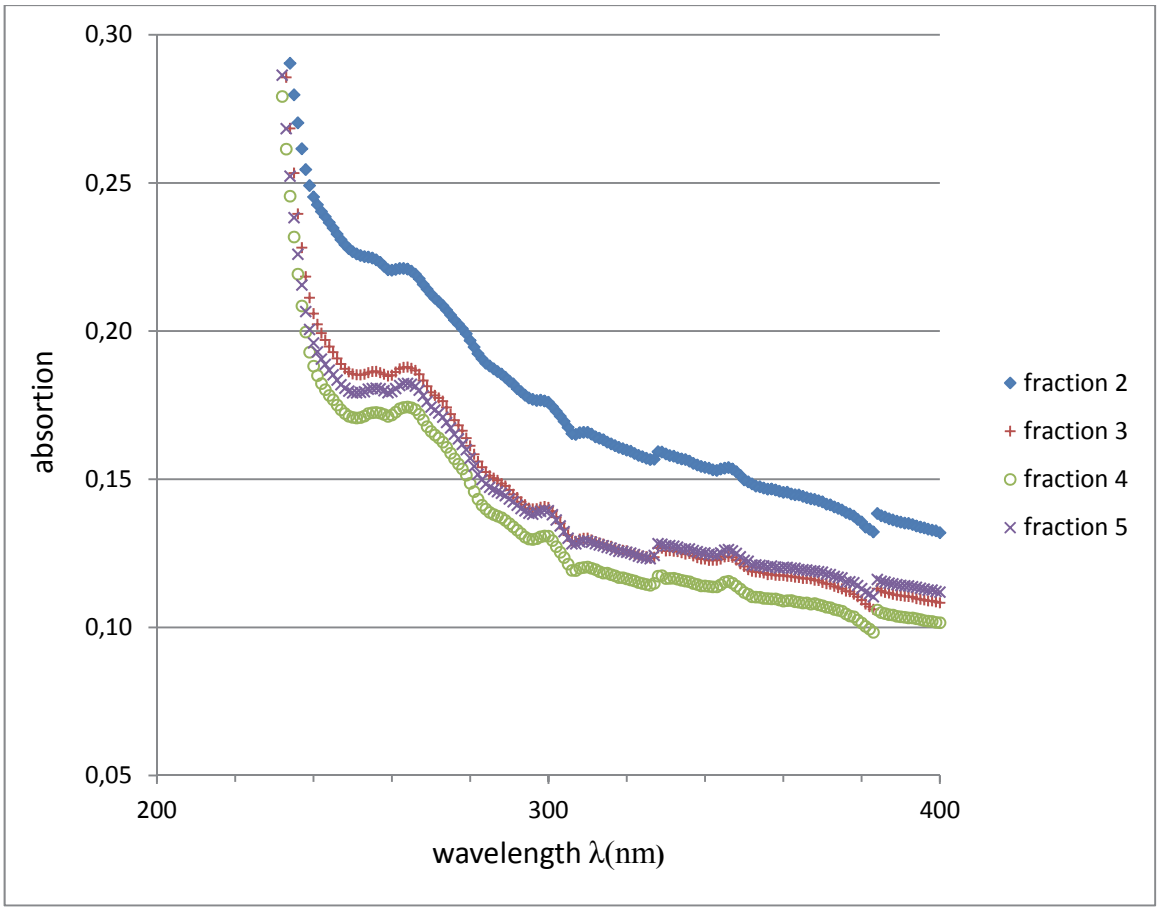

Figure 3.2 *Absorbance of purified rhodopsin by elution fraction. Data for fraction 1 was removed since the accuracy of the measurement was not satisfactory for such a low absorption.*

An estimate of the concentration of rhodopsin in the elution fractions was calculated from absorption measurement at the wavelength of maximum absorption using the Lambert-Beer law [67]

$$A = \varepsilon c d, \qquad (1)$$

where absorption of a liquid denoted by A equals to its molar concentration c [mol/l] multiplied by measurement distance d [cm] and extinction coefficient ε [$cm^{-1}(mol/l)^{-1}$].

From equation (1) we deduct the molar concentration

$$c = A / \varepsilon d. \qquad (2)$$

By multiplying the molar concentration with the molecular weight M [g/mol] we get the concentration c [g/l]. The extinction coefficient for rhodopsin is 55 810 $cm^{-1}(mol/l)^{-1}$ and its molecular weight is 38 893 g/mol. [68]

The absorbance peak for rhodopsin was observed near 265 nm instead of the expected 280 nm. Consequently absorbance values at maximum wavelength were used in

determining the concentrations, in order to maintain coherence with other measurements.

Table 3.4 *Maximum absorption and deducted concentration for elution fractions of rhodopsin.*

elution fraction	λ_{max} (nm)	A at λ_{max}	c (µg/ml)
2	265	0,22019	153,63
3	266	0,18668	129,90
4	266	0,17347	120,96
5	264	0,18234	127,18

Before an exact concentration assay, the relative concentrations of the elution fractions of purified IFNγR1 were also assessed with absorbance spectroscopy at 280 nm. The difference in absorbance of the fractions shows the relative amounts of purified protein present in each fraction.

Table 3.4 *Maximum absorption for elution fractions of IFNγR1.*

elution fraction	λ (nm)	A at 280 nm
1	280	0,036
2	280	0,066
3	280	0,101
4	280	0,081
5	280	0,066

3.2.4.1 BCA assay

The exact concentration of purified IFNγR1 was measured with a BCA assay using a Pierce® BCA Protein Assay Kit from Thermo Scientific. This colorimetric assay uses cupric sulfate together with bicinchionic acid (BCA) which is a stable and highly specific to cuprous ion. It is a relatively fast protein assay and only requires one reaction step, as BCA is stable in alkaline conditions. It is also tolerant to many common interference agents, particularly detergents such as SDS and Tween-20, which can be tolerated up to 1% concentration without reducing the sensitivity of the assay, although reducing agents, EDTA and strong acids might noticeably interfere with the result. [69]

The reaction takes place in two steps. In the first step two temperature dependent reactions occur: at room temperature cysteines, tyrosines and tryptophans reduce Cu^{2+} ions from the cupric sulfate to Cu^{+}-ions, while between 37°C to 60°C also the peptide bonds in protein participate in the reducing reaction. The resulting amount of Cu^{+} ions is in proportion to the amount of protein present in the solution, and when performed at higher temperatures it is less sensitive to the percentage of aromatic amino acids in the examined protein. [69]

In the second step two BCA molecules react with Cu^{+}-ion forming a chelate complex that produces a purple color. The intensity of the color is then measured with a spectrophotometer, normally showing as a strong absorbance peak at 562 nm. The intensity of absorbance at 562 nm is nearly linear with increasing protein concentration within the range 20-2000 µg/ml. [69]

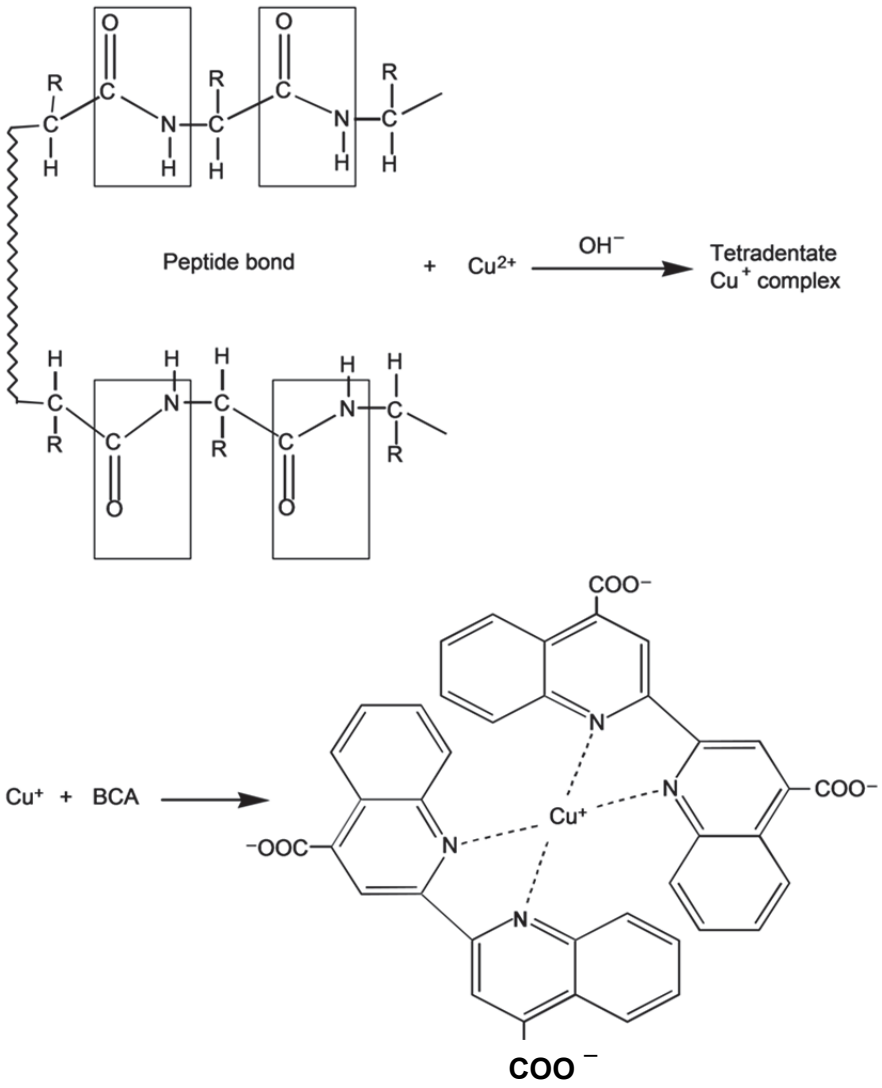

Figure 3.3 *Reaction of cupric sulfate with protein peptide bonds at alkaline conditions produces a tetradentate Cu^{+}-complex. The following reaction of copper-treated protein with BCA causes the formation of purple color, the intensity of which is then determined by measuring the consequent absorption peak at 256 nm.* [69]

The absorption maxima for the standards as well as the measured samples appeared at 528 nm instead of the expected 562 nm, and 526 nm was thus used for preparing the standard curve and calculating the concentration of the samples. This may be due to the realized incubation time exceeding the recommended 30 minutes, which is likely to have caused the observed shift in the wavelength of maximum absorption.

Table 3.5 *Absorption (A) of calibration solutions of different concentrations {c(µg/ml)}. The measurement was performed twice to prove reproducibility and confirm that the absorption maxima (λ_{max}) had indeed shifted to 526 nm.*

	1st measurement				**2nd measurement**			
c(µg/ml)	**A at 526nm**	**A at 562nm**	**λ_{max} (nm)**	**A at λ_{max}**	**A at 526nm**	**A at 562nm**	**λ_{max} (nm)**	**A at λ_{max}**
0,5	-0,00012	0,00057	**572**	0,00089	-0,00871	-0,00517	**682**	-0,00240
1,0	0,01164	0,00586	**525**	0,01172	-0,00056	-0,00121	**534**	-0,00043
5,0	0,01584	0,00989	**521**	0,01591	0,00000	0,00000	**572**	0,00000
10	0,05065	0,02675	**528**	0,05065	0,03989	0,02007	**526**	0,03998
25	0,04247	0,02264	**524**	0,04255	0,03633	0,01900	**526**	0,03645
50	0,09792	0,05149	**526**	0,09801	0,08550	0,04339	**526**	0,08563
125	0,27653	0,14346	**526**	0,27691	0,23487	0,11800	**527**	0,23500
250	0,59249	0,30970	**526**	0,59347	0,50591	0,25709	**527**	0,50623
sample 1	-	-	**-**	-	0,02922	0,01403	**526**	0,02922
sample 2	-	-	**-**	-	0,00937	0,00426	**526**	0,00937

The measurement appeared not to be sensitive enough for concentrations of less than 5,0 µg/ml which can be seen in table 3.5 as negative values after baseline correction. This was expected as the recommended working range for this assay starts from a 20 µg/ml concentration. The calibration curves are thus calculated based on absorption values for concentrations of 5,0 µg/ml and higher.

The calibration curves were drawn for both measurements at 562 nm, at 526 nm and at λ_{max}. The two last ones coincided with each other, showing that the absorbance maxima had shifted consistently for all samples with 36 nm. The absorbances at 562 nm were noted to be considerably lower than for a typical calibration curve for BCA assay with a BSA standard.

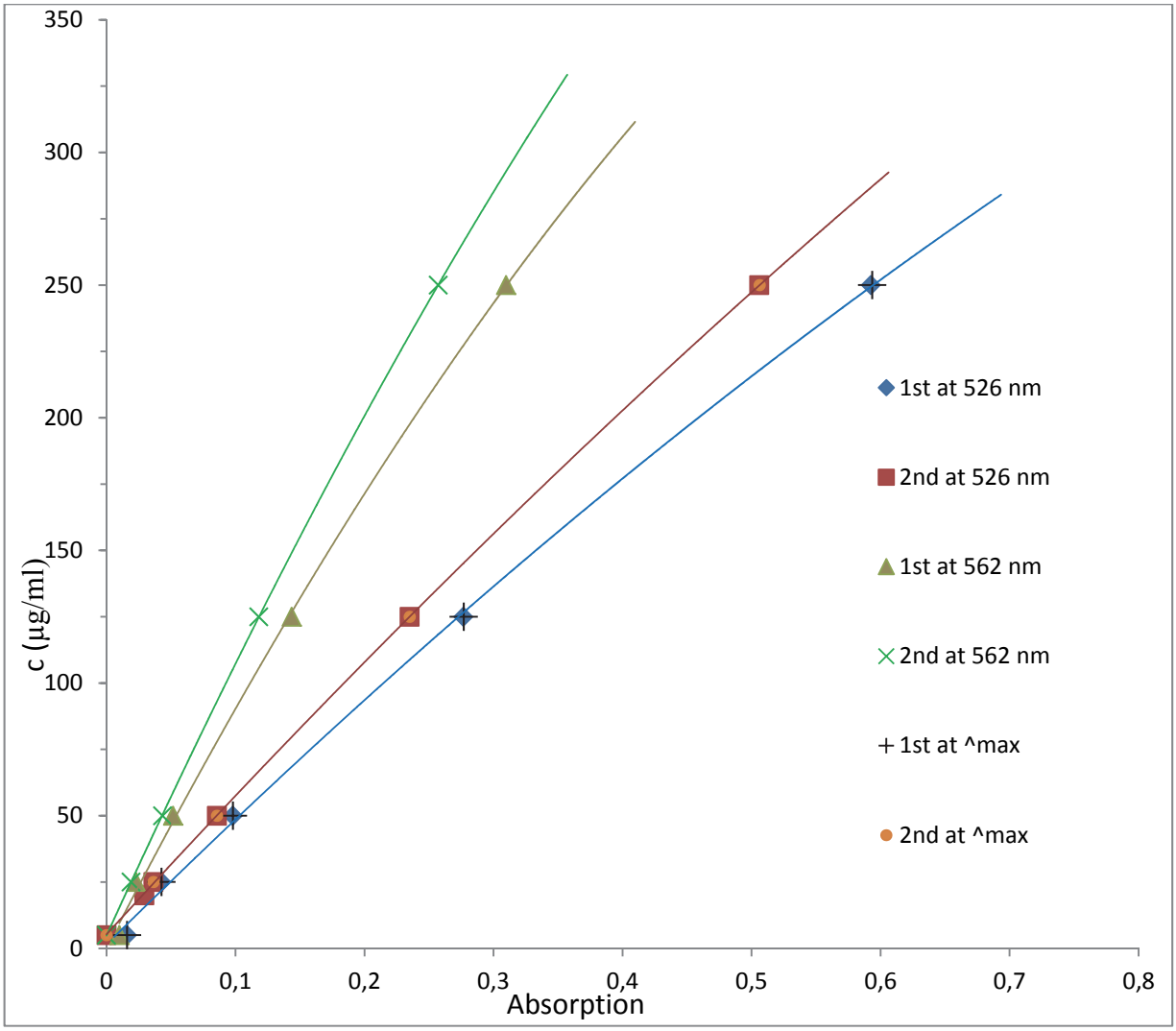

Figure 3.4 *Calibration curves for both measurement sets displaying relation of concentration and absorption both at the expected peak at 562 nm and the observed peak at 526 nm.*

Calibration at 526 nm was selected as a base for determining the protein concentration of unknown samples. The calibration curve of the second measurement was used in calculating sample concentration, as the second measurement was conducted within a shorter amount of time and with a shorter delay from measurement of the samples, and was thus considered more reliable.

The concentration of sample 1 was calculated to be 20 µg/ml and the concentration of sample 2 10 µg/ml. However, due to nonlinearity of the function below 20 µg/ml and the instability of the measurement at low concentrations, the result for sample 2 was discarded as unreliable.

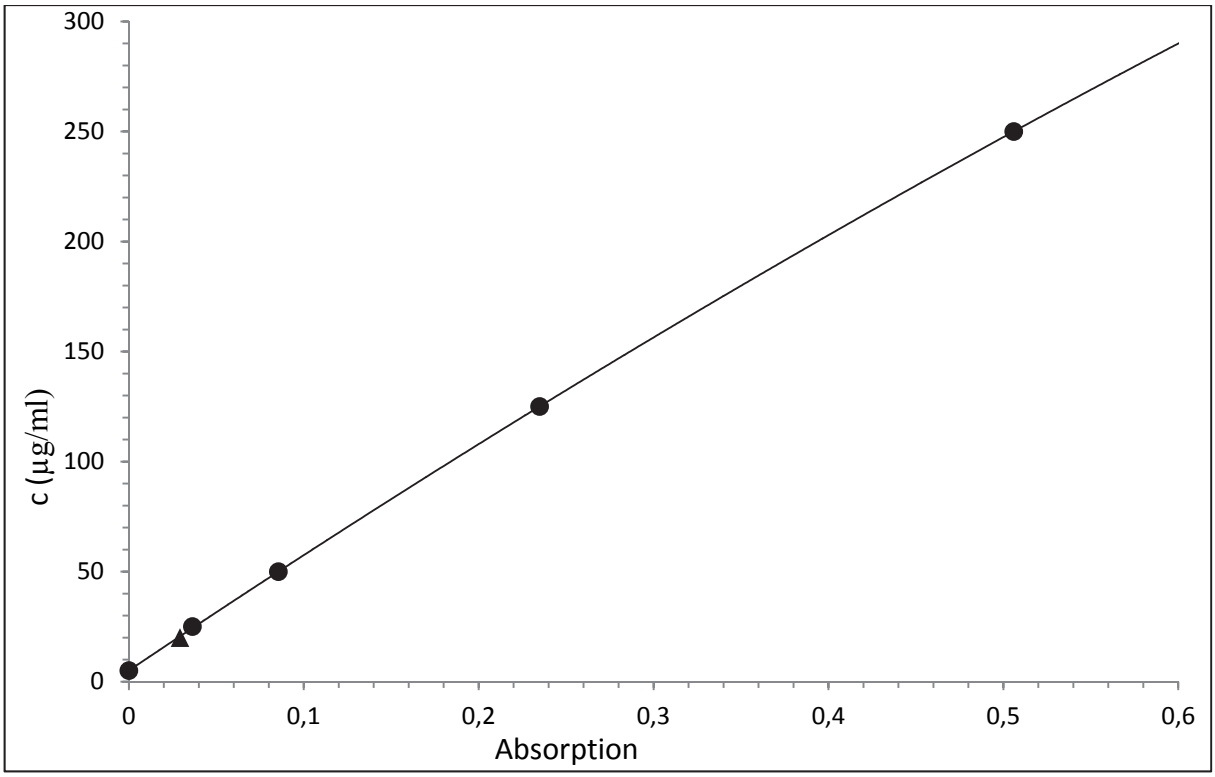

Figure 3.5 *The position of sample 1 of IFNγR1 at the calibration curve at 526 nm.*

3.3 Characterization of proteins

3.3.1 SDS-PAGE

The identity of expressed and purified proteins was assessed with gel electrophoresis. SDS polyacrylamide gel electrophoresis (PAGE) is widely used in biochemistry to separate proteins by their electrophoretic mobility, which describes the relation of a polypeptide chain and its charge. Sodium dodecyl sulfate is used to convey a negative charge on the polypeptide chain, hence linearizing the chain by charge repulsion. The binding of SDS on the denatured protein usually spreads an even charge per unit mass, thus enabling separation of proteins by their mass when exposed to an electric field.

As tertiary and quaternary protein structures are formed by several different mechanisms, additional measures are often taken to linearize the protein. Heat may be used for disrupting protein folding, and commonly either DTT or mercaptoethanol is added to break disulfide bonds. Covalently attached carbohydrate and phosphate groups may be difficult to remove and might affect the final molecular weight of the denatured protein. This makes SDS-PAGE too insensitive for accurate molecular weight determination but it remains a reliable indicator of the presence of purified proteins. [13]

The concentration of the polyacrylamide gel is determined by the molecular weight range in which the examined proteins are expected to appear. The higher the molecular weight, the lower does the acrylamide percentage of the gel need to be in order to pass through the heavy molecules.

Table 3.6 *Sizes of studied proteins according to UniProt Knowledgebase* [68]

Protein	Rhodopsin	IFNγ	IFNγR1	IFNγR2	JAK1	Caspase-1
Weight (Da)	38 893	19 348	54 405	37 806	133 277	45 159
Length (AA)	348	166	489	337	1 154	404

All the proteins in this study were within the range of 15-55 kDa, except for JAK1, which has a molecular weight of 133 kDa. A 10% polyacrylamide resolving gel separates well proteins within 10-100 kDa, and was considered sensitive enough for all of the proteins in question.

In order to prevent the spreading of protein molecules horizontally in the resolving gel, the protein micelles are first run through a lower percentage polyacrylamide gel named stacking gel. Due to lower polymer concentration the gel has a larger pore size allowing protein micelles to fall faster and rapidly stack on top in each other.

Glass plates were carefully assembled in the casting frame and checked for possible leakage with water. The 10% polyacrylamide resolving gel was prepared in a 50 ml tube with purified water, 1,5 M Tris at pH 8,8, 10% SDS, 30% acrylamide, Temed and 10% ammonium persulfate (APS) according in proportions described in the SDS-PAGE protocol (appendix 4). The gel solution was poured between the tight glass sandwich directly after adding polymerization catalyzing Temed and APS. A 2 mm layer of water was poured on top of the gel to smoothen the edge of the gel. The resolving gel was let to polymerize for approximately 30 minutes.

The 4% polyacrylamide stacking gel was prepared in a 25 ml tube with the same ingredients as for the resolving gel, but using 1,5 M Tris at pH 6,8 and different proportions to obtain a lower polymer concentration (appendix 4). The stacking gel solution was poured on top of the polymerized resolving gel and the plastic comb was slid between the glass plates avoiding formation of bubbles in the gel. The stacking gel was allowed to polymerize for another 30 minutes, while the samples were prepared for loading. Sample preparation differed according to the proteins in question, but for loading all samples were mixed with one part of 3x Chaps loading buffer and two parts of the sample protein solution (appendix 4).

Once the stacking gel was polymerized, the glass plate sandwich was placed in the clamping frame in electrode assembly which was filled with running buffer consisting of Tris-base (solid), glycine, 10% SDS and purified water (appendix 4). The plastic combs were carefully removed leaving sample wells in the gel intact and the samples were pipetted in the wells. The electrodes were connected and electricity was run through the gel at 100 V for 60 minutes.

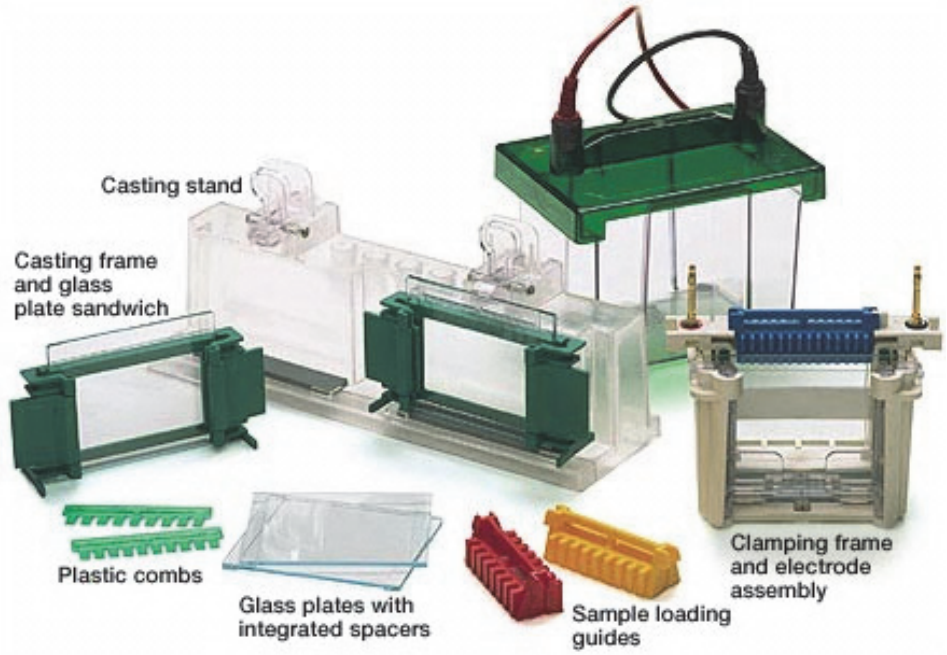

Figure 3.6 *SDS-PAGE equipment used in the study.* [70]

After running the electricity for 60 minutes, the gel was carefully removed from the class plates and washed 3 times for 5 minutes in 100 ml of purified water. For visualizing the protein bands the gel was incubated in 50 ml of Coomassie Blue stain at room temperature. The non-binding stain was then washed off twice for 60 minutes in 100 ml of purified water. All washing and staining was performed in end-over-end mixing to ensure even treatment over different protein bands.

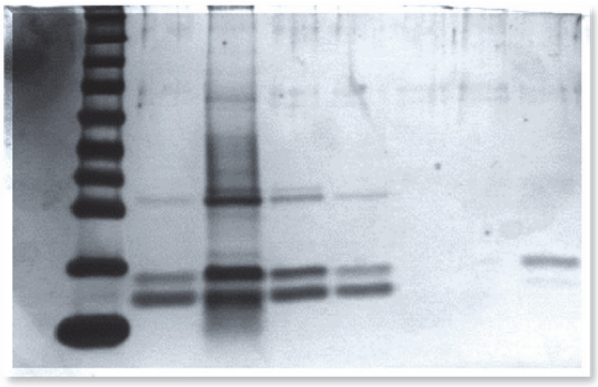

Figure 3.7 *Example of an SDS-PAGE gel stained with Coomassie Blue. The marker ladder on the left indicates approximate sizes of protein with smaller molecules falling further down in the gel.*

3.3.2 Western Blot

Western blotting is a protein analysis technique in which proteins are first separated by size and then identified with specific antibodies. Due to the latter phase of the technique it is sometimes also called immunoblotting. SDS-PAGE can be first used to separate desired proteins from an unpurified cell lysate, or simply to position proteins as distinguished bands on the gel from a purified solution.

After electrophoresis the proteins are transferred from the gel to a nitrocellulose or polyvinylidene difluoride membrane. The gel is placed on top of the transfer membrane and electrical current is run through it causing the protein to migrate from the gel to the membrane and thus producing a replica of the gel's protein pattern adhered on the membrane. The membrane is then incubated in some general protein such as milk protein in order to block non-specific binding of antibody to the membrane outside the protein bands.

The protein of interest is then probed with a specific antibody, which is linked to a reporter enzyme that can be detected with chemiluminescence or fluorescence agents. A more traditional method is to use a two-step process in which the membrane is first probed with a primary antibody specific to the target protein and a secondary antibody, which carries the reporter enzyme. The two-step process is more time consuming but may facilitate detection of small amounts of protein since several secondary antibodies bind to one primary antibody thus enhancing the signal. A one-step process only involves one antibody, which both recognizes the target protein and carries a reporter enzyme. [8]

The plasmids for all the studied recombinant proteins were prepared with a Strep-tag which could be probed with a one-step Strep-Tactin® Horseradish peroxidase (HRP) conjugated antibody from IBA GmbH. The horseradish peroxidase cleaves the introduced chemiluminescence agent producing chemiluminescence in proportion to the amount of detected protein.

To confirm the identity of the purified proteins a western blot was performed after running an SDS-PAGE. Instead of staining, the gel was placed on a nitrocellulose membrane between two extra thick filters papers on the platinum anode of a transfer apparatus. The filters were pre-soaked in transfer buffer consisting of Tris-base, glycine and 100% methanol. Bubbles were removed by rolling a sterile pipette on the filter stack and the cathode plate was placed on top. Electrical current was connected and run through at 15 V for 60 minutes to transfer proteins to the nitrocellulose membrane. A Bio-Rad Trans-Blot® SD Semi-dry Electrophoretic Transfer Cell was used for all western blots in the study.

After transfer the membrane was incubated in blocking agent consisting of milk protein in wash buffer (appendix V) for 60 minutes. The membrane was washed three times for 5 minutes with wash buffer. 10 µl of Strep-Tactin antibody was diluted in 10 ml of wash buffer and the membrane was incubated for 60 minutes. Non-bound antibody was rinsed off by washing the membrane 4 times for 1 minute with was buffer before developing chemiluminescence reaction. All operations with the membrane were performed in end-over-end mixing at room temperature.

For visualizing the results a Thermo Scientific Pierce ECL Western Blotting Substrate was used, which is a peroxidase substrate for enhanced chemiluminescence (ECL) for detecting horseradish peroxidase activity. 2 ml of enhancer solution was mixed with stable peroxide buffer in 1:1 ratio and directly applied to the membrane for one minute. The membrane was then drained and placed between plastic protector sheets before developing an autoradiograph image.

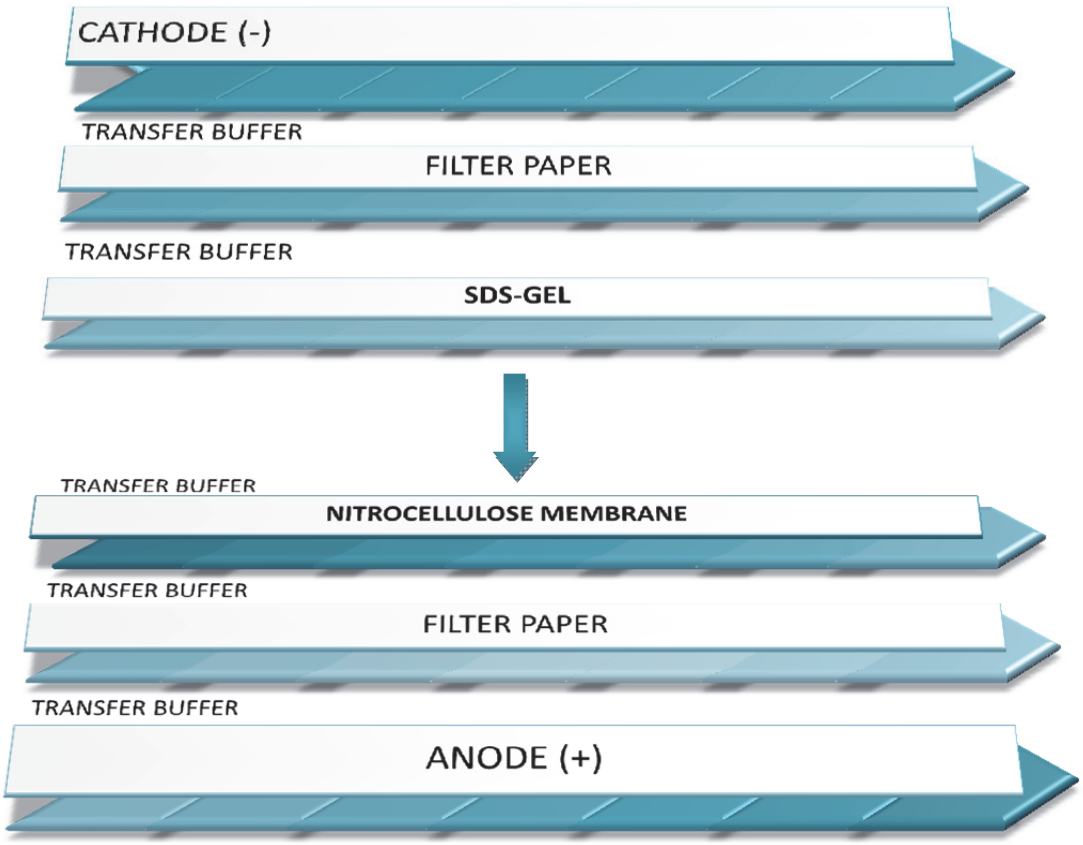

Figure 3.8 *Assembly of a semi-dry electrophoretic transfer stack.*

In the process of establishing a stably expressing HEK-293 cell line with IFNγR2, all the 56 initial monoclonal cell cultures were screened for target protein expression by western blotting with the Strep-Tactin® HRP conjugated antibody. Based on the

expression strength observed on the blot, the best clones were selected for expansion of cell cultures. Once the expanded cultures were confluent, they were screened again and finally 5 best expressing clones were selected for inoculating suspension flasks for high-yield protein production.

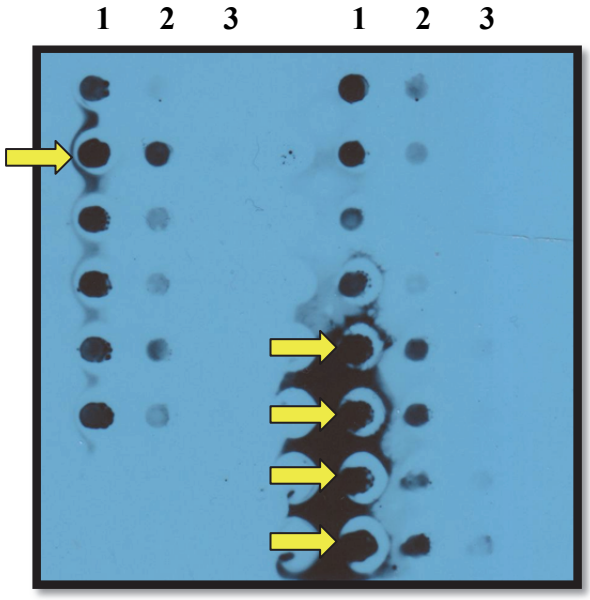

Figure 3.9 *Western blot image of the second screening for IFNγR2 expression. The dots in columns number 1 represent the undiluted samples, while column 2 is for 5-fold dilution of sample in column 1 and column 3 a 5-fold dilution of sample in column 2. The clones pointed with arrows were selected for cell culture expansion.*

3.3.3 Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is a technique based on magnetic properties of atomic nuclei. When the nuclei of certain atoms are immersed in a static magnetic field and exposed to a second oscillating magnetic field, they resonate at a characteristic radio frequency. The frequency is determined by the spin of the nucleus, which needs to be different from zero in order to be detectable. The most commonly used atoms in NMR spectroscopy are the proton ^{1}H, ^{13}C isotope of carbon, ^{15}N isotope of nitrogen and ^{31}P isotope of phosphor. [12]

The resonance frequency of a nucleus varies slightly depending on the position of the atom within the molecule, its so-called chemical environment. Measuring this subtle variation called the chemical shift, enables determining the structure of a molecule based on the relative frequencies of the atoms in the molecule. The chemical shift is in the order of one part in a million of the characteristic resonance frequency and is given in parts per million (ppm). [12]

A one dimensional (1D) spectrum is recorded when the resonance of only one type of atom is measured. A two dimensional (2D) spectrum can be recorded by combining the measurement of chemical shifts for two different atoms, often ^{1}H with ^{13}C or ^{15}N. 1D spectra can be detailed enough for resolving structures of relatively simple organic molecules but in determining complex bimolecular structures a 2D spectrum is imperative. [12]

The most common method in protein NMR is the heteronuclear single quantum correlation (HSQC) experiment with ^{1}H and ^{15}N. Every amino acid in a protein except for proline has an amide proton attached to a nitrogen in the peptide bond (previously shown in Figure 2.2). The HSQC records the correlation between the resonances of the nitrogen and the amide proton, yielding a peak in the spectrum for each amide-group. This means that every amino acid of the protein gives an observable backbone peak in the spectrum, except for proline, which has no amide proton due to its ring structure. In addition, side chains with nitrogen bound protons will produce peaks. [71]

Assigning the HSQC spectrum requires more than the 2D experiment itself. Using triple resonance experiments with ^{1}H, ^{13}C and ^{15}N sequential connectivity between residues can be determined. Once an HSQC spectrum for a protein is reliably assigned, it can be used for detecting binding sites in protein-ligand interactions. Comparing the spectrum of a free protein with the spectrum of protein-ligand complex may show changes in location of some peaks, indicating that the chemical shifts of corresponding residues have changed due to ligand binding. [71]

4. RESULTS

4.1 Expression and purification of rhodopsin control

Rhodopsin was expressed in COS-1 cells with a well-established protocol first described by Oprian et al. [44] It was purified with 1D4-antibody affinity chromatography with 1D4 coupled Sepharose beads following a purification protocol by Hwa et al. [72]

The identity of the purified protein was characterized with a Coomassie blue stained SDS-PAGE. The five purified fractions were all found to contain a single protein band between marker lines pointing at approximately 37 kDa and 50 kDa. Fractions 2, 1 and3 show a higher protein content as is common in affinity chromatography.

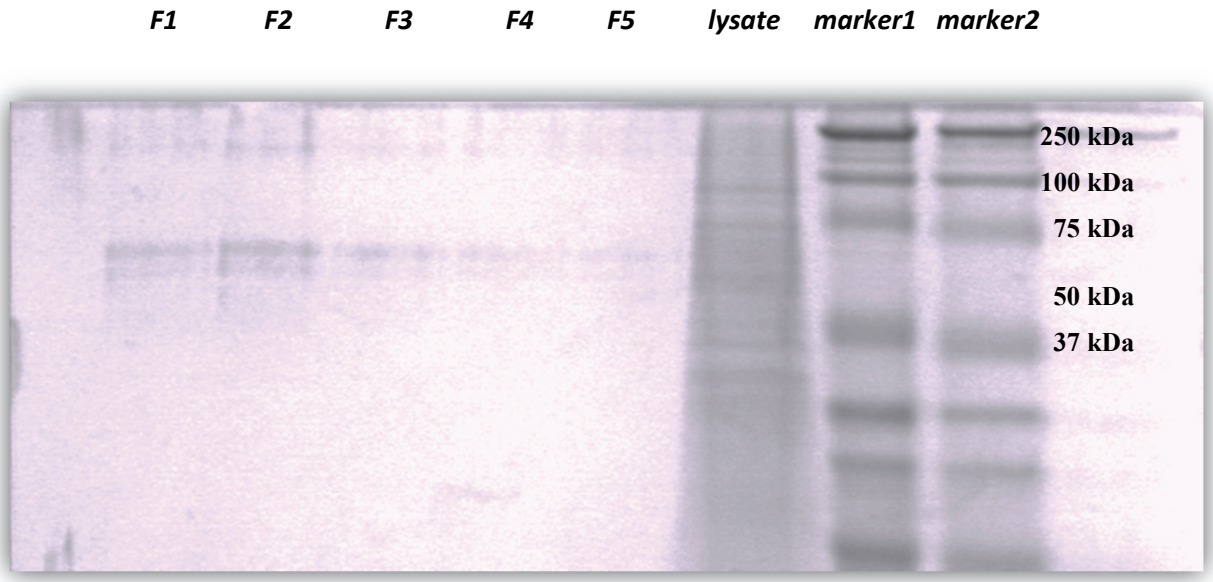

Figure 4.1 *SDS-PAGE of five fractions of purified rhodopsin (F1 to F5) together with solubilized cell extract and two markers.*

Rhodopsin has an expected molecular weight of 39 kDa. The band for the purified protein appears to be around 60 kDa, which is an unusual result. There is only one band at the same molecular weight for each fraction, and the bands decrease in strength towards later fractions, which indicates a successful purification with no unspecific binding, regardless of the unexpected band position. Rhodopsin is easily glycosylated, and the presence of N-glycans may shift the molecular weight drastically upwards. [64, 73]

4.2 Expression and purification of IFNγ receptor complex components

IFNγ and IFNγR1 were expressed with a transient transfection in COS-1 cells. Stable HEK-293 cell lines were transfected with both receptors IFNγR1 and IFNγR2 but only cells transfected with IFNγR2 survived in the antibiotic resistance selection. The related signaling mediating proteins JAK-1, JAK-2 and STAT-1 were left out of scope of the experiment, in order to simplify examining the interaction of IFNγ with its two receptors.

IFNγ, IFNγR1 and IFNγR2 plasmids all carried a Strep-tag and the respective proteins from both transient and stable transfections were all purified with Streptavidin affinity chromatography, with the protocol described in (appendix 3).

Solubilized IFNγR1 protein extracts from the harvested COS-1 cells were purified in two sets: the first one directly after harvesting and the second one a week later. The later set of protein extracts were flash frozen in liquid nitrogen and stored at -20°C directly after harvesting.

The first set of purified IFNγR1 was characterized with a Coomassie blue stained SDS-PAGE. All five elution fractions presented a protein band with a slight duplet nature approximately at 100 kDa. This is surprising considering the expected molecular weight of 54 kDa for IFNγR1.

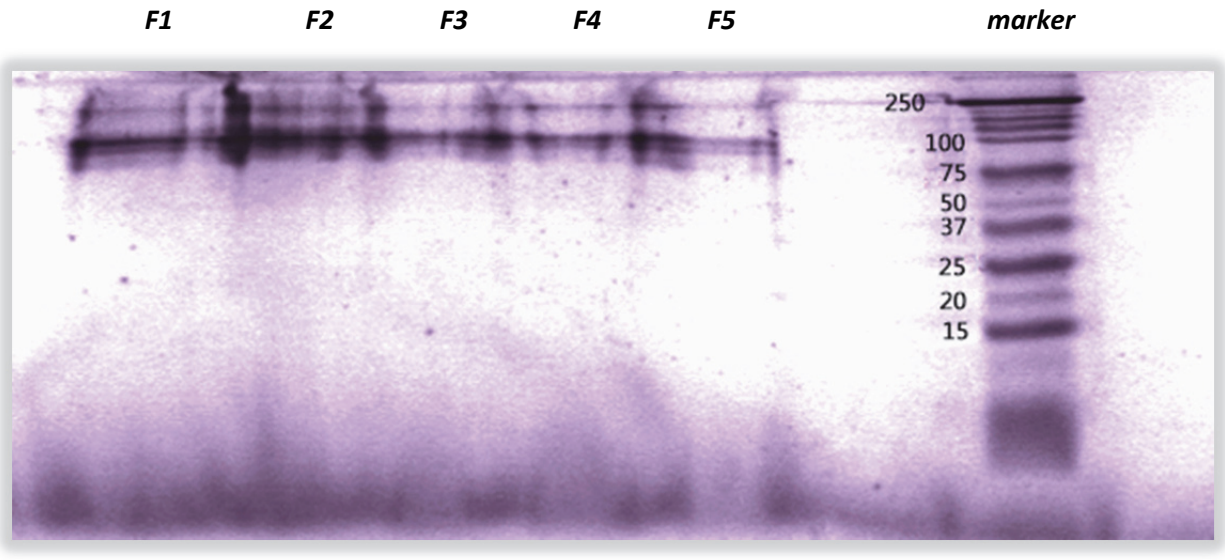

Figure 4.2 *SDS-PAGE of five elution fractions of the first purified IFNγR1 (F1 to F5) with marker bands shown in kDa.*

A second SDS-PAGE was conducted in a similar fashion, with the exception of briefly boiling the samples before loading the gel. Bringing the samples rapidly to boiling point

is a method commonly used to break multimeric composition of proteins and prevent aggregation of samples.

In this case, preparation of samples with or without boiling resulted in no difference in the size of the SDS-PAGE resolved protein. The protein bands in the second SDS-PAGE for boiled IFNγR1 samples also appeared approximately at 100 kDa. The bands were considerably weaker than for the first set of IFNγR1 but distinct.

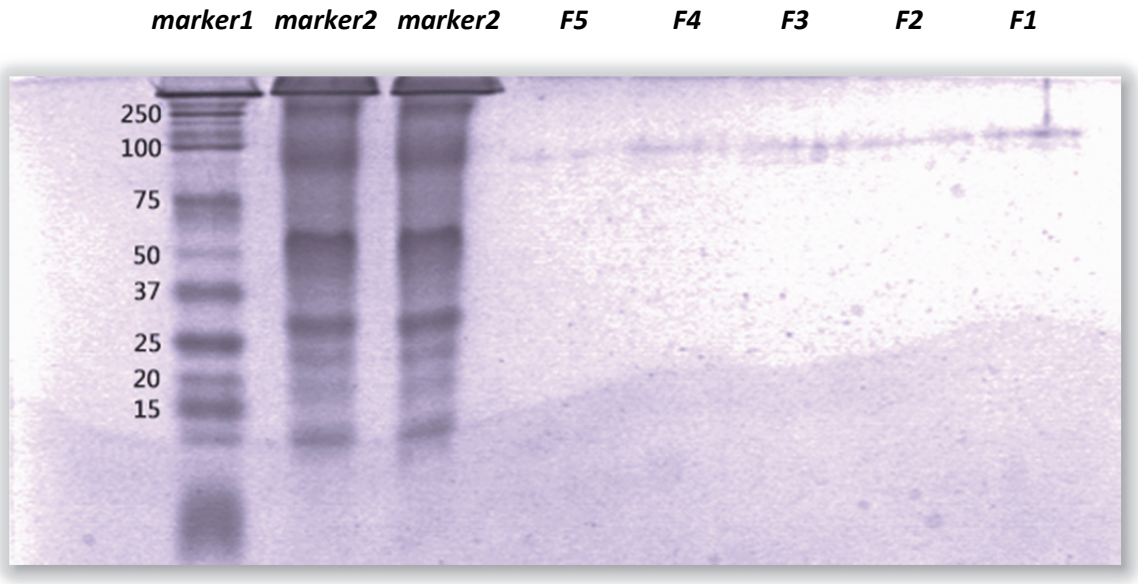

Figure 4.3 *SDS-PAGE of five elution fractions of the first purified IFNγR1 (F1 to F5) ran with briefly boiled samples.*

The molecular weight of 100 kDa at which bands for IFNγR1 appear, seems to indicate tight dimerization of the receptor. The strong resistance of a multimer towards SDS denaturing and disulfide bond reducing agents, like mercaptoethanol, is exceptional, although not unheard of.

In order to verify the identity of IFNγR1, a functional assay was conducted. The specific binding of IFNγR1 to IFNγ was assessed with commercially available IFNγ bought from Creative Biomart. The second set of COS-1 expressed IFNγR1 was co-purified with the purchased IFNγ with the same Streptavidin affinity chromatography as the previous one. To confirm the binding of IFNγ to IFNγR1, half of the IFNγR1 was eluted in five fractions with IFNγ present in the column, and half in five fractions without the ligand.

The proteins present in elution fractions for both IFNγ-IFNγR1 complex and IFNγR1 alone were then characterized by an SDS-PAGE with IFNγ alone as a reference. (Figure

4.4) The IFNγR1 bands appeared close to 100 kDa as even more distinct duplets as the bands for the first set of purified receptor.

Bands for IFNγ were observed in elution fractions 2, 3 and 4 of the complex, which were also the fractions with the highest concentrations of the receptor present. The IFNγ in the complex displayed smeared bands around 20 kDa, which is expected as the purchased protein contained IFNγ with different glycosylation states. The presence of IFNγ in the elution fractions verifies that the expressed IFNγR1 binds its ligand, as the purchased IFNγ contained a His-tag instead of a Strep-tag and thus wouldn't have been eluted without binding to the receptor.

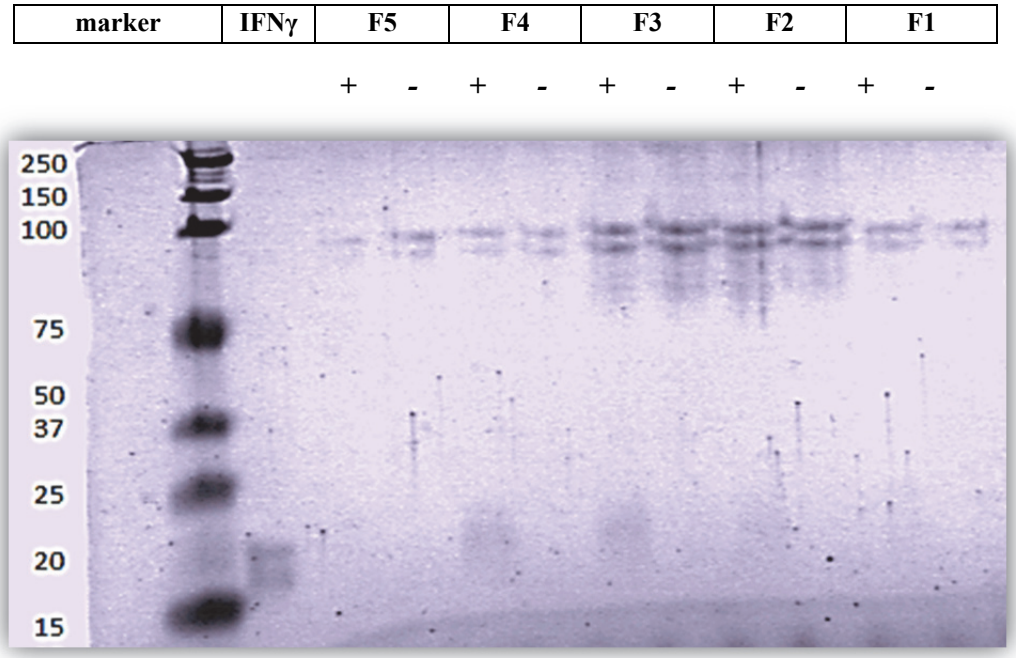

Figure 4.4 *SDS-PAGE of five elution fractions of the second purified IFNγR1 (F1 to F5) with (+) and without (-) the IFNγ ligand. The bound IFNγ shows clearly on the lanes of fractions 2, 3 and 4 for the receptor-ligand complex.*

The COS-1 expressed and Streptavidin affinity purified IFNγ was characterized with an SDS-PAGE together with putative IFNγ antibodies. Six uncharacterized antibodies against IFNγ were examined by request of a pharmacological company Materia Medica.

The SDS-PAGE presented strong bands for IFNγ close to 20 kDa. The observed molecular weight corresponds well with the predicted molecular weight of 19 kDa for an IFNγ monomer. The putative antibodies showed up as weak bands approximately at 100 kDa, with antibody 1 giving the strongest response. (Figure 4.5)

The bands for IFNγ appear as distinct duplets instead of one consistent band. This might be due to cleavage of the Strep-tag, different glycosylation patterns, or the combination

of both. In addition to the strong band at the predicted molecular weight, there was also a weaker duplet band for each IFNγ slightly above 35 kDa. The band presumably presents the remnants of IFNγ dimer, which has not depolymerized in the gel.

This is supported by a previous notion that IFNγ forms a very strong dimer. Using infrared spectroscopy, an aggregation band has been found for recombinant human IFNγ at 1620^{-1} cm, which has been shown to be a characteristic band length for thermally induced, or lyophilization (freeze-drying) induced protein aggregation. [74]

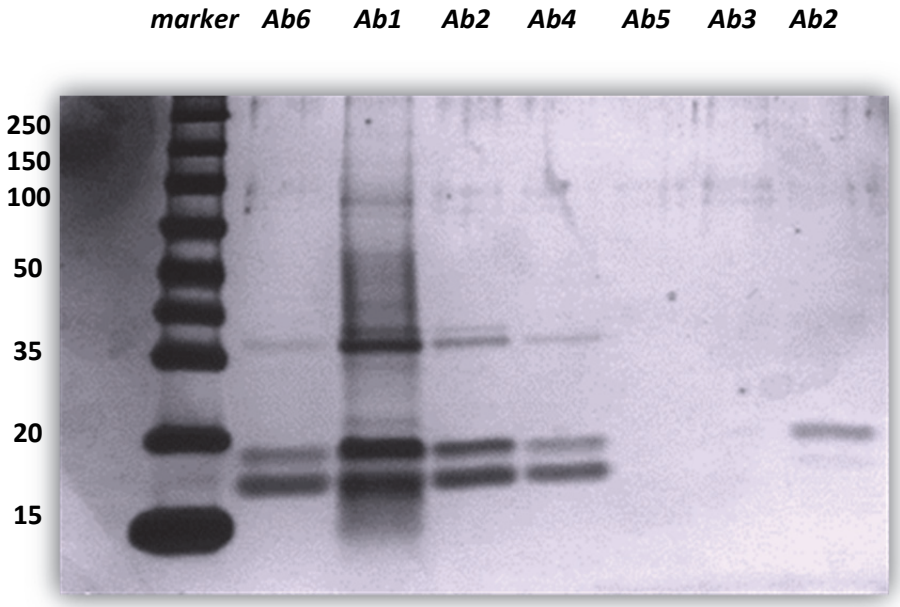

Figure 4.5 *SDS-PAGE of IFNγ with putative antibodies from Materia Medica. IFNγ shows clear bands at the lanes of antibodies 1, 2, 4 and 6. Samples with antibodies 3 and 5 did not contain IFNγ. Sample with antibody 1 also contained commercially bought IFNγR1 appearing as a spread band between 30 and 50 kDa.*

In order to conduct the following NMR experiments with non-aggregating IFNγR1 protein, some commercially available extracellular IFNγR1 was bought. The purchased protein was assessed in the SDS-PAGE together with IFNγ and antibody 1.

The protein bought from Sino Biological Inc. contained the extracellular domain of IFNγR1 consisting of Met1 – Gly245 of the IFNγR1 sequence and had a predicted molecular weight of 27,3 kDa. Due to heavy glycosylation, it was expected to appear on an SDS-PAGE as a thick spread out band between 35 and 40 kDa.

The extracellular IFNγR1 was observed as a rather smeared but distinguishable band between 30 and 50 kDa. The position and appearance of the band correspond to the expected, although it is spread over an even wider molecular weight range than predicted. (Figure 4.6)

IFNγR2 was expressed by a tetracycline inducible stable transfection of HEK-293 cells. The cell cultures were expanded and grown in suspension flasks in order to produce IFNγR2 in milligram scale. Three out of eight flasks were contaminated and the cells from five surviving were harvested for purification.

Since each flask contained a single clone of IFNγR2 expressing cells, the clones were harvested and purified separately. The cell pellets were weighed, and solubilized proteins extracted from each pellet were purified with Streptavidin affinity chromatography.

Purified IFNγR2 from two best expressing clones was characterized with SDS-PAGE. The protein bands for IFNγR2 appeared close to 100 kDa, which was not expected for the receptor with predicted molecular weight of 38 kDa.

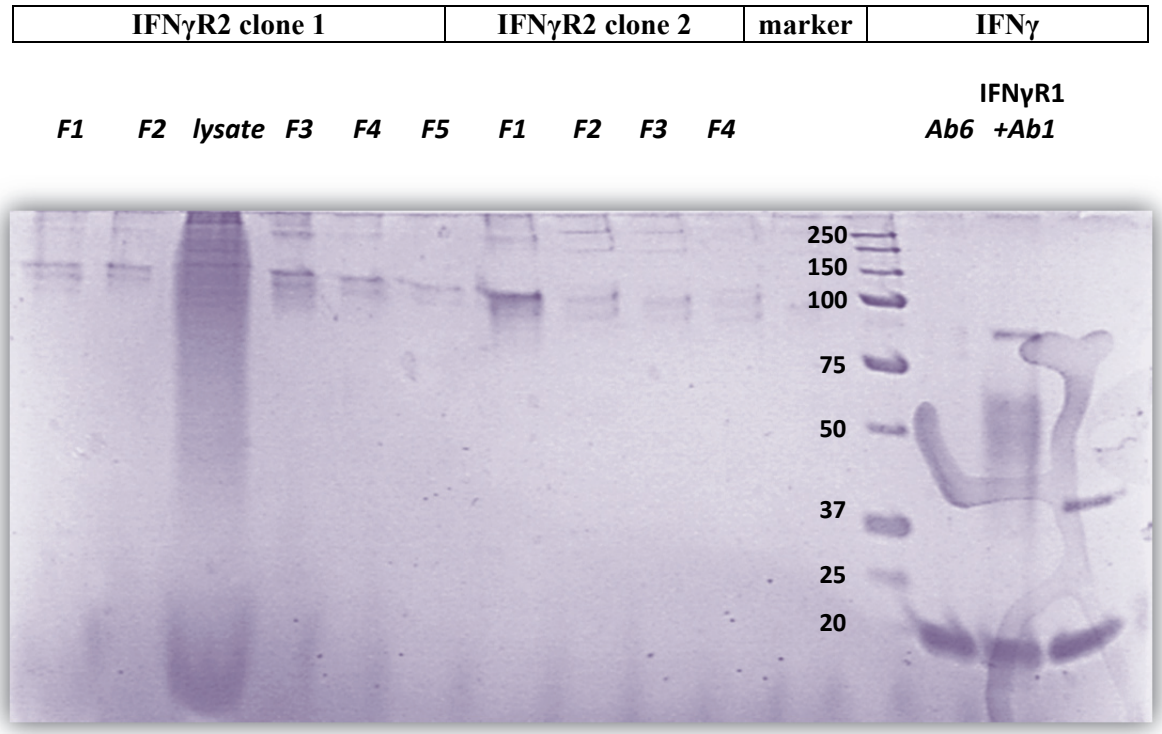

Figure 4.6 *SDS-PAGE of purified clones 1 and 2 of IFNγR2, together with IFNγ antibody experiment. The lysate and elution fractions of IFNγR2 clone 1 are on the left and elution fractions of clone 2 in the middle. On the right of the marker are IFNγ with antibody 6, IFNγ with antibody 1 + IFNγR1 and finally IFNγ alone.*

The high apparent molecular weight of the purified IFNγR2 is surprising. Dimerization of the receptor is plausible and would support the hypothesis that the secondary receptor binds to the intermediate 1:2 IFNγ-IFNγR1 complex. However, the observed molecular weight is higher than the predicted weight for an IFNγR2 dimer, which raises questions

on the glycosylation pattern and other unknown post-translational modifications of the protein.

An additional experiment was conducted on the same SDS-PAGE to examine whether antibody binding affects the dimerization and depolymerization of IFNγ. Three samples of IFNγ were loaded on the gel: one with IFNγ alone, another with IFNγ and antibody 6, and third with IFNγ-IFNγR1 complex and antibody 6. (Figure 4.6)

The sample lane for IFNγ alone displays two bands; a thick one at the expected molecular weight close to 20 kDa and a thinner one at the molecular weight for IFNγ dimer around 40 kDa. The sample lanes for IFNγ with antibodies only present a band for IFNγ at the molecular weight of the monomer but not for the dimer. The bands for IFNγR1 and antibody 1 are clear, but unfortunately the band for antibody 6 is barely visible, thus decreasing confidence in the result of the experiment.

4.3 NMR analysis of IFNγ receptor complex

4.3.1 1D NMR analysis of IFNγ, IFNγR1 and antibody interactions

The expressed IFNγ was finally characterized with NMR spectroscopy. The measured spectra both in 1D and 2D were compared with a 2D NMR spectrum for human IFNγ by Grzesiek et al. [75] The used reference displays all backbone peaks for uniformly ^{15}N labeled recombinant human IFNγ in a 2D ^{15}N - ^{1}H HSQC spectrum. The study of Greziek et al. was conducted with IFNγΔ10, which is a high biological activity form of IFNγ lacking 10 last C-terminal amino acids.

At first a 1D ^{1}H spectrum was recorded for IFNγ alone with a 900 MHz spectrometer. Only a few amino acids can be unambiguously assigned in the 1D spectrum for a complex protein, while more detailed analysis requires measurement of 2D spectra. (Figure 4.8) However, the 1D spectrum gives an overview of the profile of IFNγ and confirms feasibility of further NMR studies.

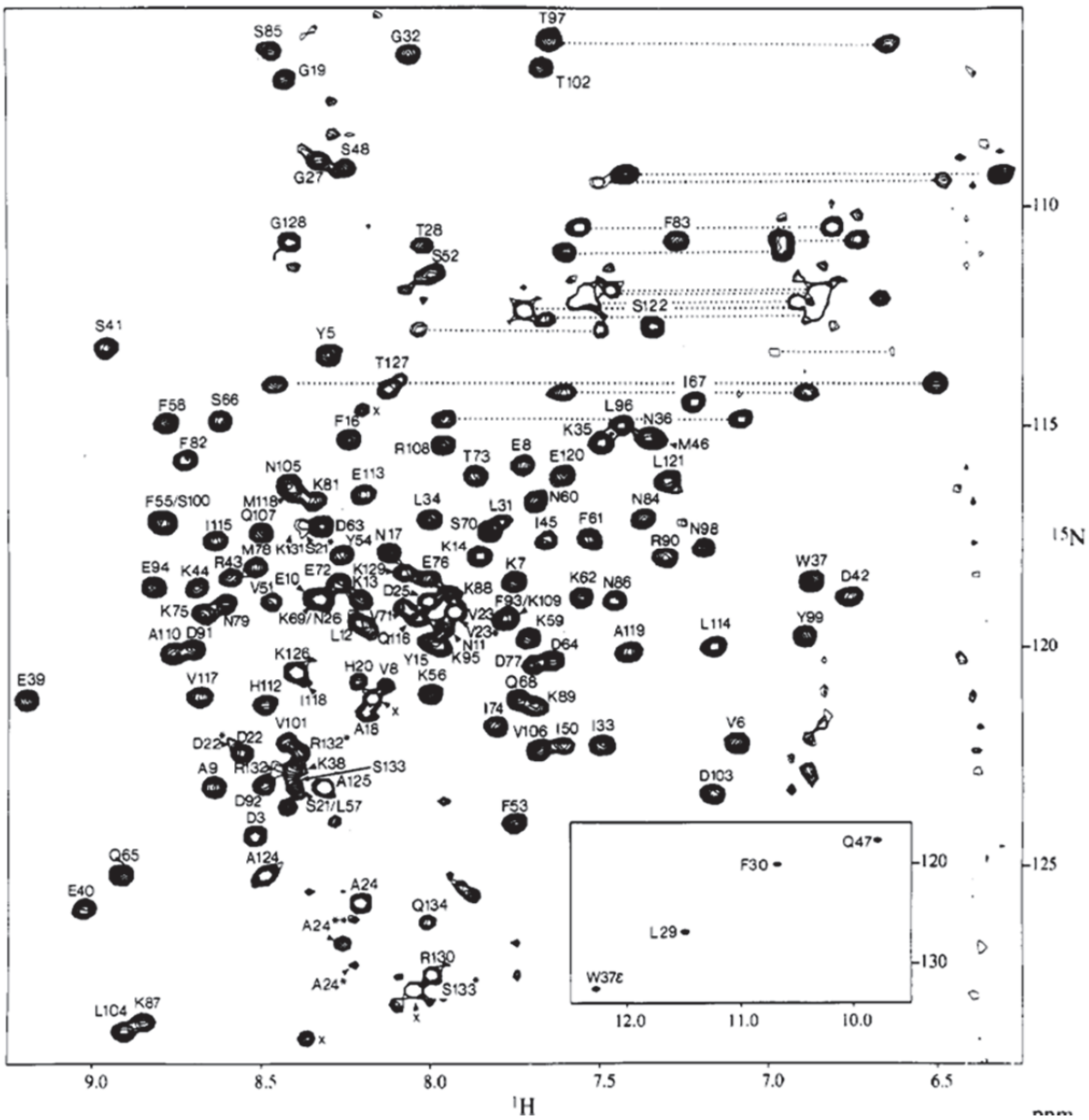

Figure 4.7 *The 2D ^{15}N - ^{1}H HSQC spectrum with labeled backbone amino acids used as a reference in NMR studies of IFNγ.* [75]

The peaks, which could be assigned, were glutamine 47 (Q47), glutamic acid 39 (E39), tyrosine 99 (Y99), tryptophan 37 (W37) and aspartic acid 42 (D42). All of these amino acids are situated close to each other in the helices, except for Y99, which is in the loop region.

W37 and E39 are located in helix B, while D42 and Q47 are near in helix C near the bend to helix B. (Figure 4.9) All of them are situated at the dimer interface of the two domains and could possess a role in dimerization interactions. Y99 is located in the loop between helices E and F but is also possibly in position to interact with residues in the C helix of the other domain.

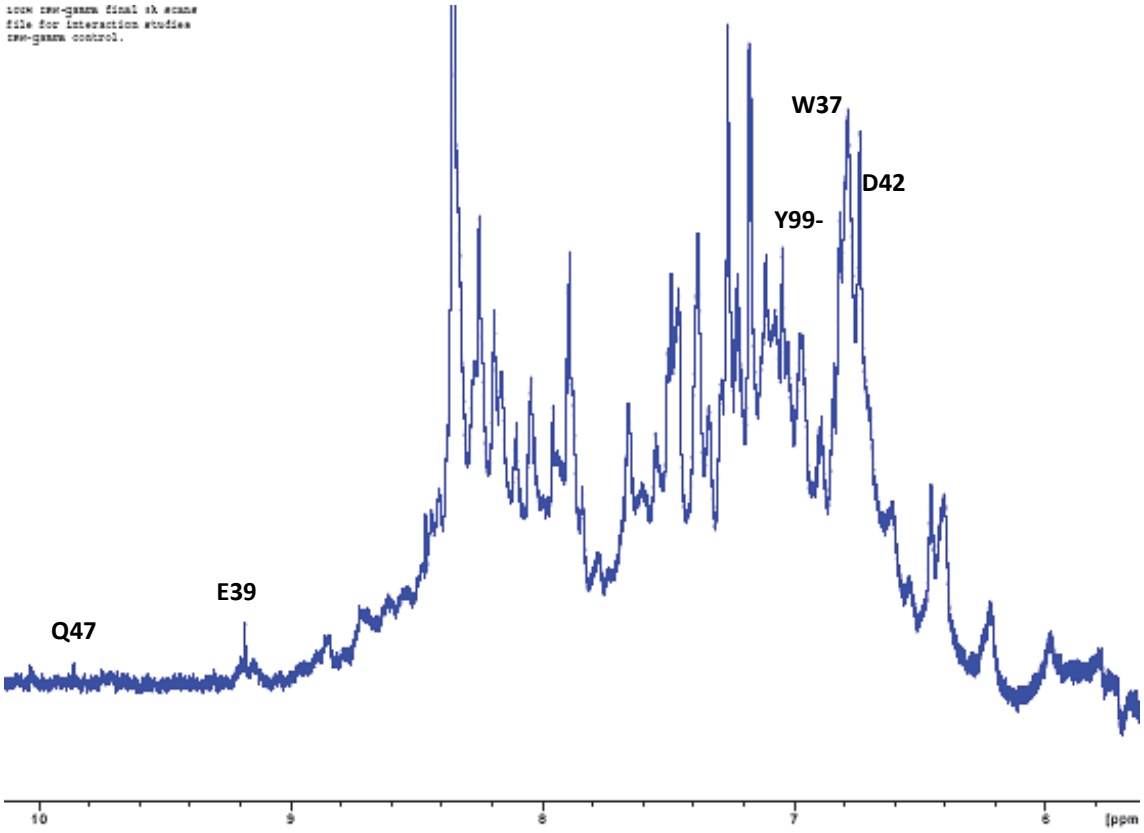

Figure 4.8 *^{1}H NMR spectrum of IFNγ. Five of the side peaks could be assigned, while resolving rest of the protein requires 2D spectra.*

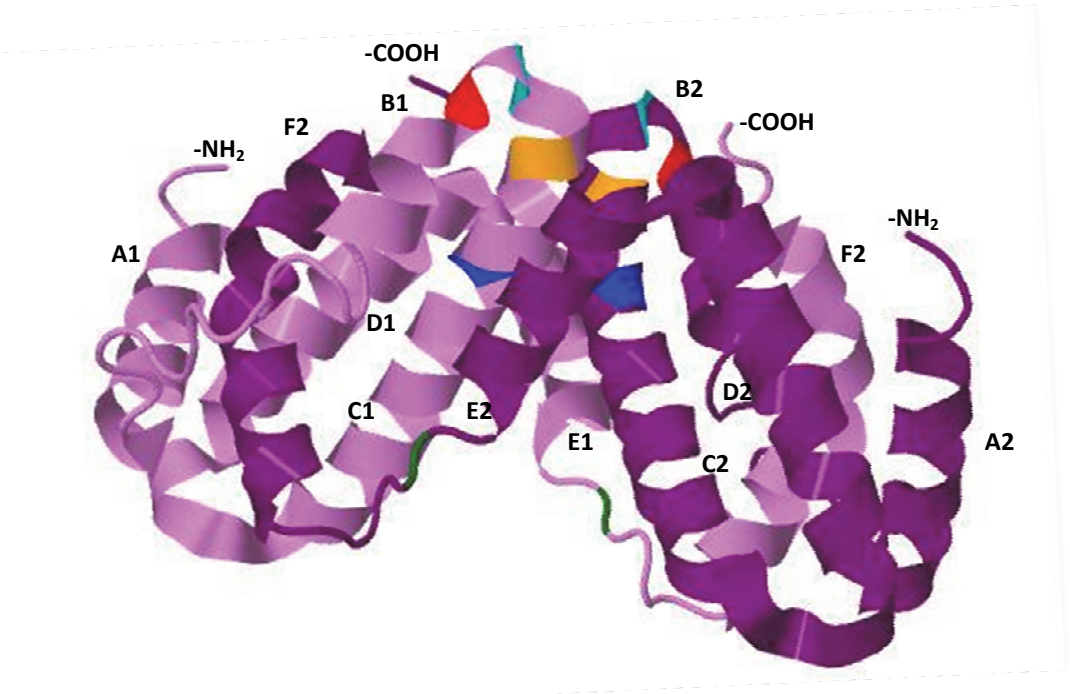

Figure 4.9 *A cartoon representation of IFNγ dimer with labeled helices and residues assigned in the 1D spectrum. Y99 is presented green, while Q47 is blue, D42 orange, E39 cyan and W37 red.*

A second NMR experiment was run in 1D for IFNγ in complex with IFNγR1. Upon addition of the receptor, the spectrum became in general broader which is likely to be due to the large size of the receptor protein. Several peaks disappeared and three entirely new peaks appeared in the spectrum. (Figure 4.10)

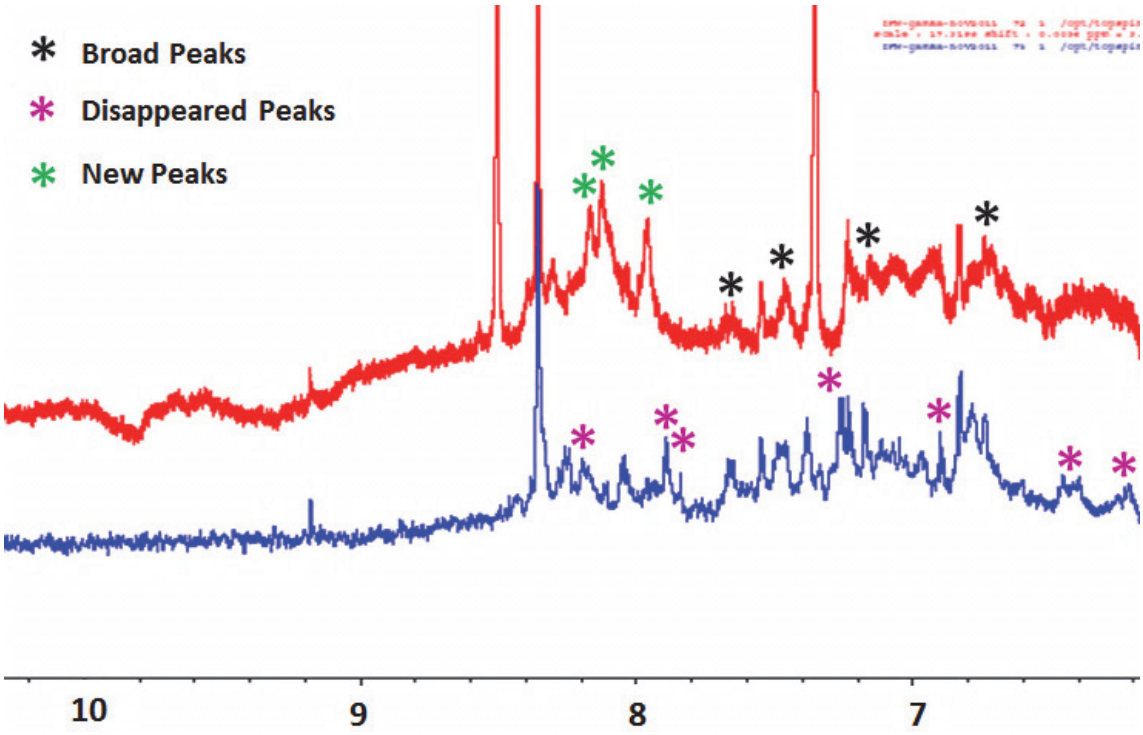

Figure 4.10 *^{1}H NMR spectrum of IFNγ with IFNγR1 (red) in comparison with spectrum IFNγ alone (blue). The spectrum became broader, three new peaks appeared and several peaks disappeared.*

The complete disappearing of many peaks indicates that the receptor molecules shade a large surface area of the ligand. This is coherent with the knowledge of IFNγ forming a complex with its primary receptor in 1:2 stoichiometry, and the observation of attachment of even a third receptor molecule. The appeared new peaks could not be assigned to specific residues considering the resolution of 1D spectrum.

The next step was to measure a 1D spectrum for IFNγ-IFNγR1 complex together with antibodies. Antibody 1 was chosen as a model as it had presented the strongest signal on the SDS-PAGE, while other antibodies only showed weak bands.

The spectrum for the ligand-receptor-antibody complex turned out very broad, preventing any reasonable analysis on the interaction. (Figure 4.11) This might also be due to the large size of the antibody, which together with the receptor forms a large complex surface that cannot be examined in detail with only 1D spectra.

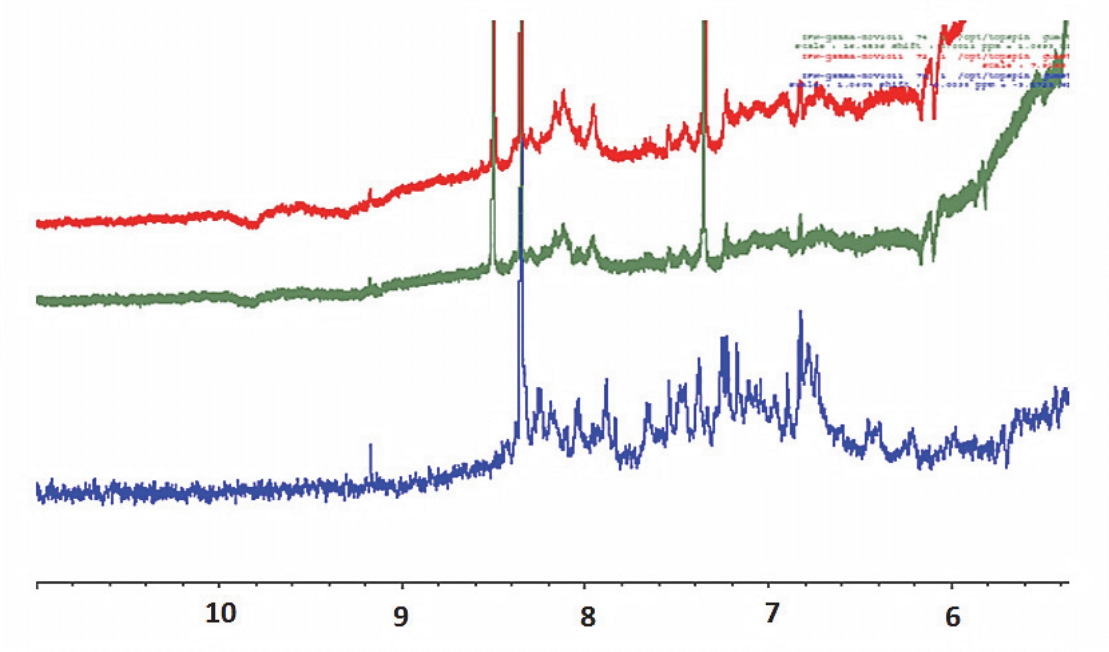

Figure 4.11 *^{1}H NMR spectrum of IFNγ with IFNγR1 and antibody 1 (green) in comparison with the spectra of IFNγ with IFNγR1 (red) and IFNγ alone (blue). Addition of the antibody to the ligand-receptor complex even further broadened the spectrum to the extent that no analysis was possible on the nature of the interaction.*

Since the study of IFNγ, IFNγR1 and antibody 1 together didn't appear feasible, a spectrum was recorded for IFNγ with antibody 1. (Figure 4.12) If the binding of antibody to IFNγ disrupted the dimer as suspected, the absence of the receptor should not hinder the effect of the antibody to the ligand. The spectrum turned out sharp and several changes in peaks were recorded.

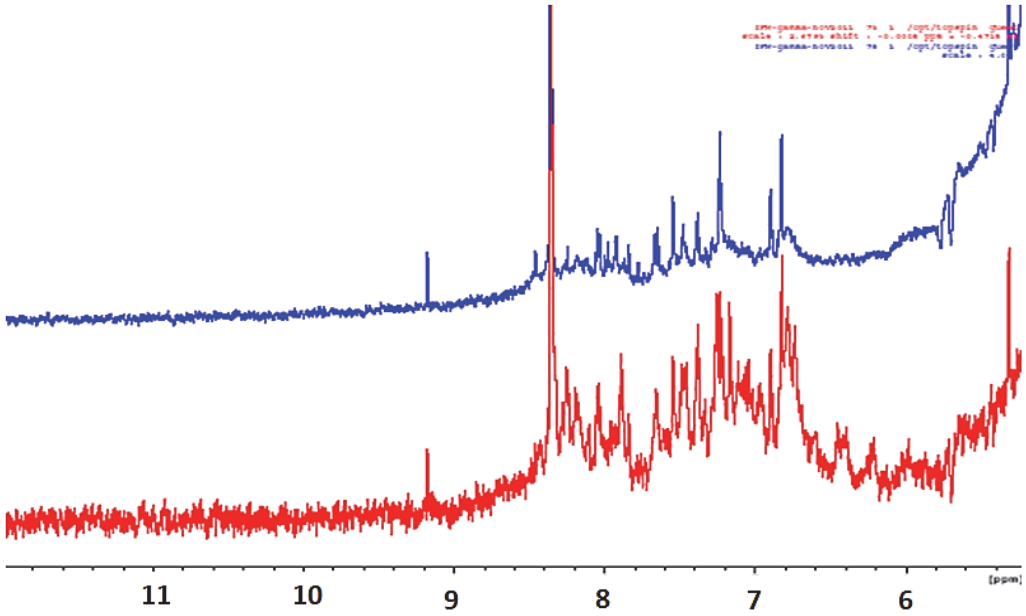

Figure 4.12 *^{1}H NMR spectrum of IFNγ with antibody 1 (blue) in comparison with the spectra of IFNγ alone (red).*

As with the spectrum of IFNγ-IFNγR1 complex, also now several peaks disappeared completely and some new peaks were observed, but the overall broadening of the spectrum was more moderate. In the region of most backbone peaks between 6,5 and 8,5 ppm, four new peaks had appeared. These new peaks could not be caused by the antibody since the spectrum of the antibody alone had not shown similar peaks. Thus, the new peaks must be due to conformational changes in IFNγ.

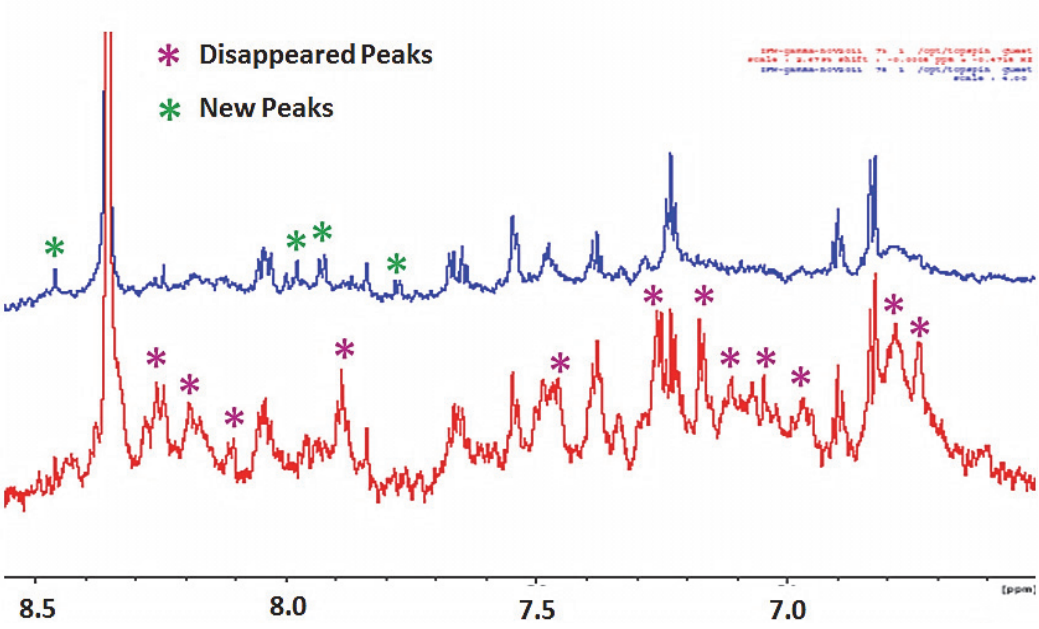

Figure 4.13 *^{1}H NMR spectra of IFNγ with antibody 1 (blue) and IFNγ alone (red). In the region of most backbone peaks four new peaks appeared and several disappeared.*

While a few peaks disappeared and new ones appeared, most of them remained unchanged or only turned slightly broader. This indicates that the binding of antibody to IFNγ is specific and targets certain residues at a determined binding site.

When the spectra of IFNγ alone and with antibody 1 were compared at the same signal to noise ratio, the presence of the antibody seemed to increase the intensity of peaks. Some of the peaks with a major increase in intensity were ones identified in the spectrum of IFNγ alone: E39, W37 and D42. (Figure 4.14)

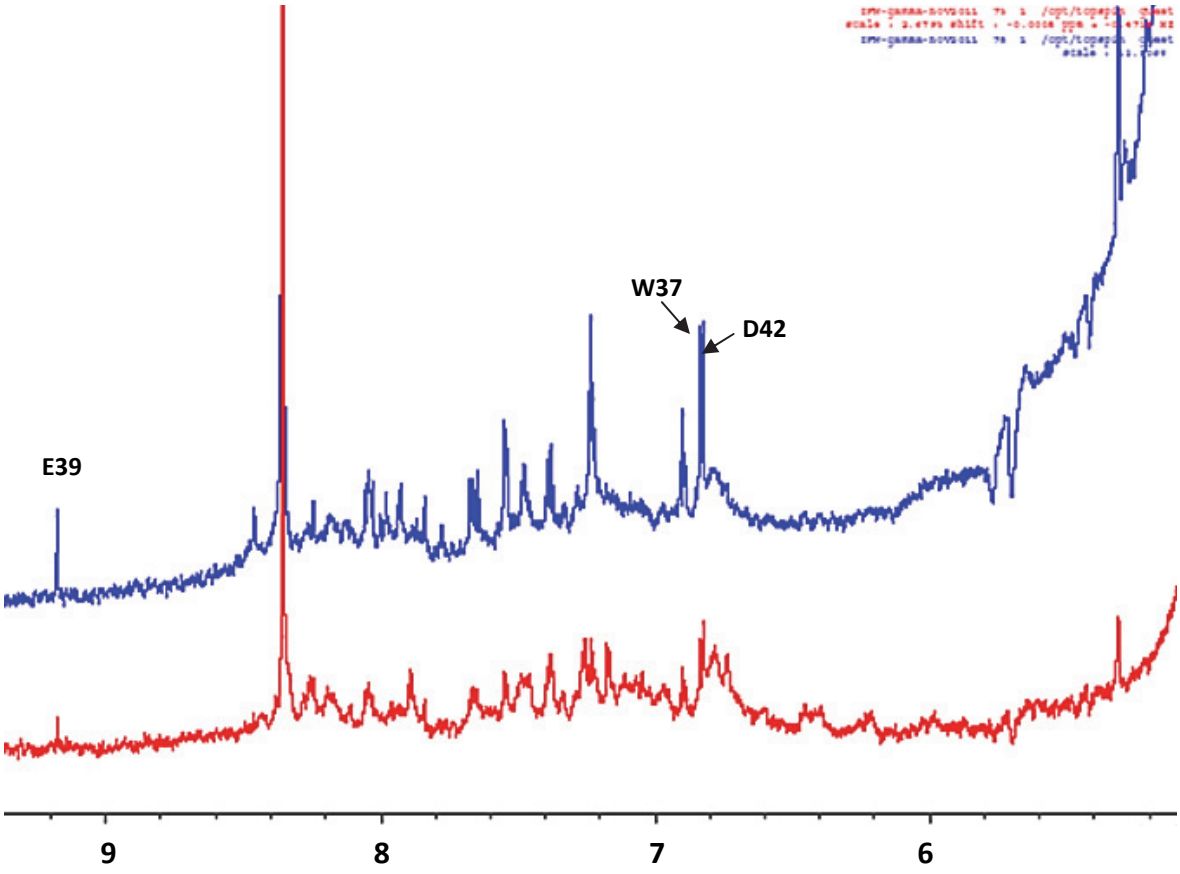

Figure 4.14 *^{1}H NMR spectra of IFNγ with antibody 1 (blue) IFNγ alone (red) with identical signal to noise ratio. The addition of antibody increased signal intensity in general and specifically for identified peaks E39, W37 and D42.*

The three identified peaks with increased intensity upon antibody binding are all situated close to the bend between helices B and C at the center of the dimer interface of IFNγ. The location indicates that due to the interaction with the antibody, residues which have been shaded at the interface of the IFNγ dimer become more available for NMR response. This enforces the hypothesis that antibody 1 affects IFNγ via disrupting the dimer formation.

4.3.2 2D NMR analysis of IFNγ, IFNγR1 and antibody interactions

A 2D ^{1}H - ^{15}N HSQC spectrum was recorded with a 900 MHz NMR spectrometer first for IFNγ alone and then for IFNγ with antibody 1. The backbone peaks for IFNγ alone were assigned in the spectrum with the reference from Greziek et al. Nearly all the peaks for backbone amino acids could be assigned unambiguously, while a few remained slightly too broad for certain assignment.

The spectrum for IFNγ with antibody 1 was recorded in the same conditions as for IFNγ alone. The same general observation was made concerning the effect of antibody

presence as in the case of 1D spectra; the spectrum became sharper and the peaks more intense. The effect of antibody binding was examined in detail both by superimposing the spectra for IFNγ alone and with the antibody, and by comparing the IFNγ-antibody spectrum with the reference.

While analyzing the two recorded spectra together with the reference it was observed that in total 16 peaks had shifted after addition of antibody, out of which 8 peaks had changed location remarkably and 8 others had shifted slightly in position.

Table 4.1 *Changes is IFNγ peaks after addition of antibody 1 with color indicators for Figure 4.15.*

Slightly shifted peaks	E8	G19	A24	L34
	S52	K56	S122	Q134
Strongly shifted peaks	F16	E39	E40	D42
	I74	F82	V117	A119

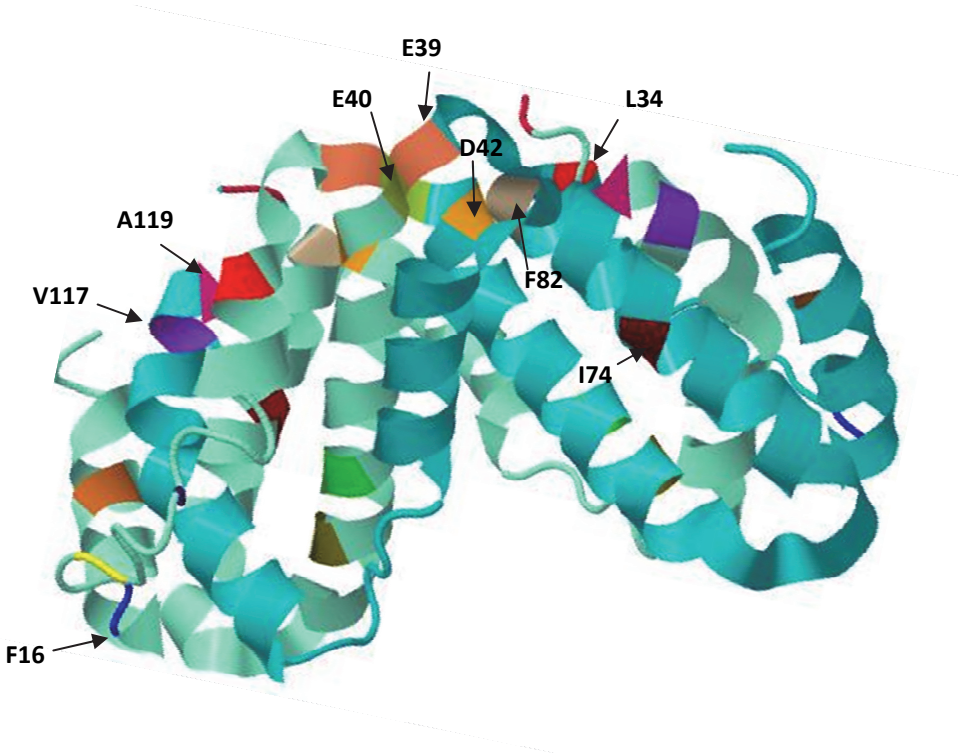

Figure 4.15 *IFNγ dimer with strongly shifted peaks labeled. Residues E39, E40, D42, F82, V117 and A119 are clearly positioned at the dimer interface and likely to take part in oligomerization.*

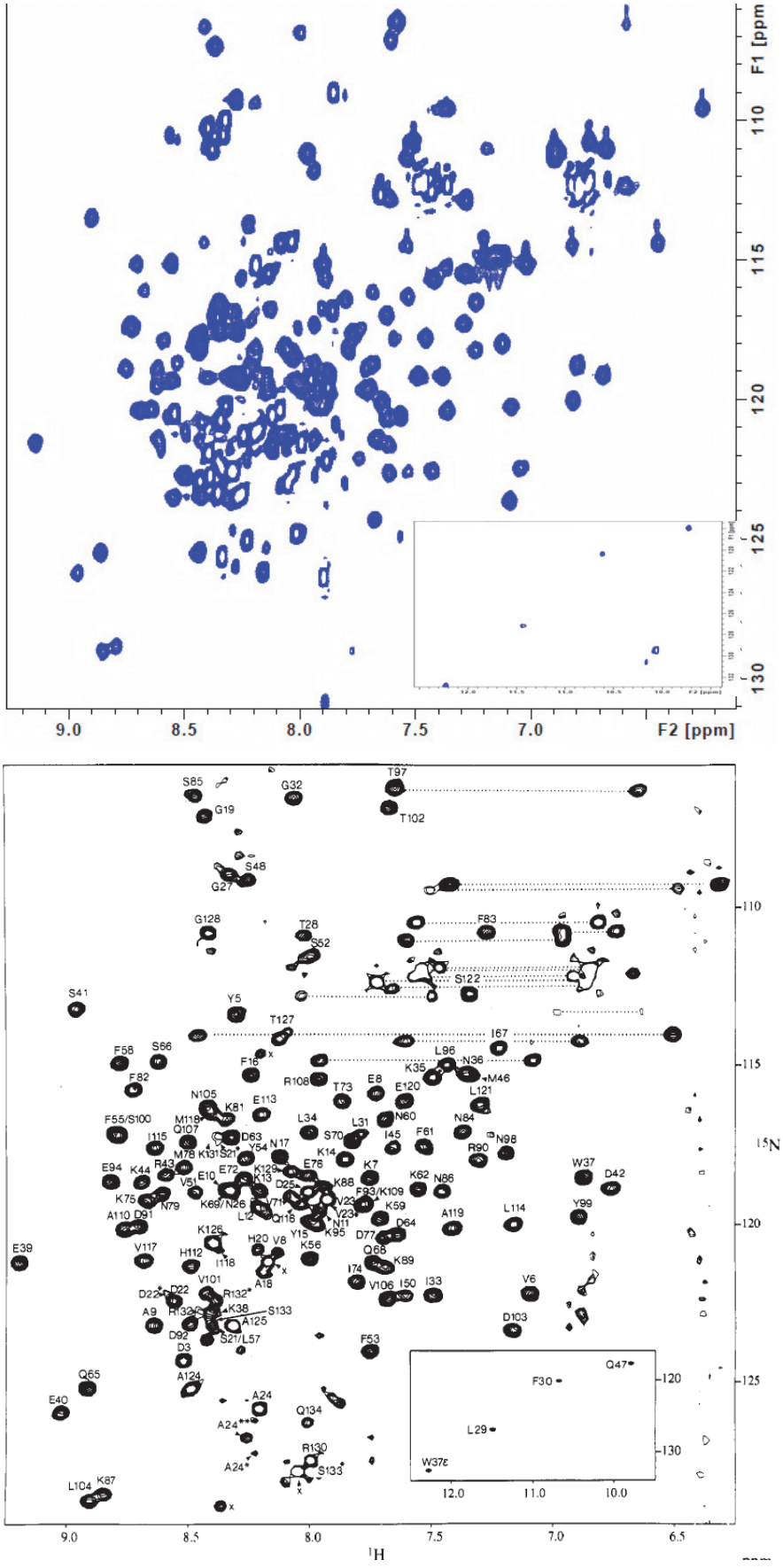

Figure 4.16 *Comparison of the recorded 2D spectrum for IFNγ with the reference. The recorded spectrum is a contour stripped version for clarity of general shape of spectra.*

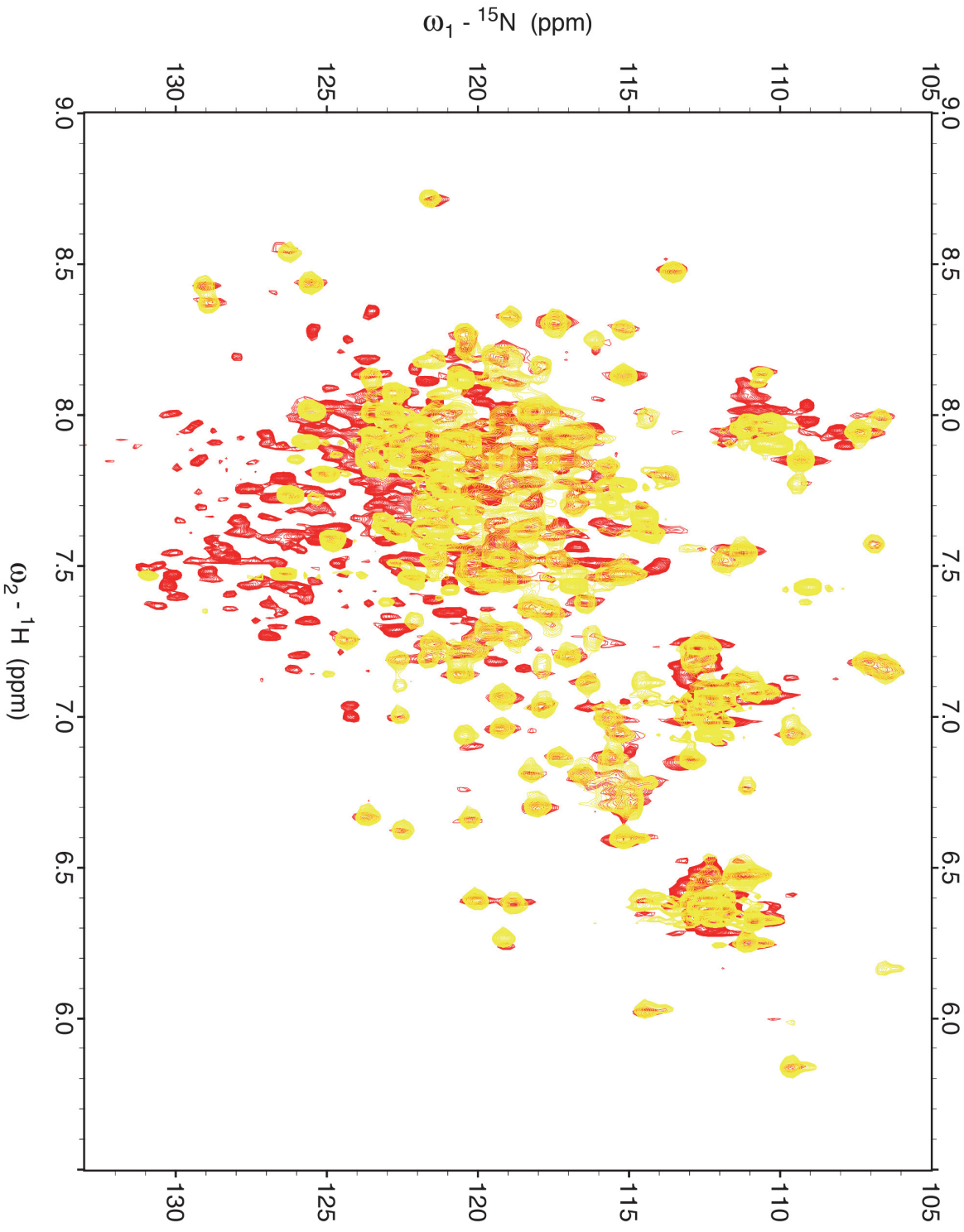

Figure 4.17 *The recorded 2D spectra for IFNγ alone (red) and IFNγ with antibody 1 (yellow) overlapping.*

The clearly shifted peaks in the IFNγ-antibody1 spectrum were identified as glutamic acid 8 (E8), phenylalanine 16 (F16), glycine 19 (G19), alanine 24 (A24), leusine 34 (L34), glutamic acid 39 (E39), glutamic acid 40 (E40), aspartic acid 42 (D42), serine 52 (S52), lysine 56 (K56), isoleusine 74 (I74), phenylalanine 82 (F82), valine 117 (V117), alanine 119 (A119), serine 122 (S122) and glutamine 134 (Q134).

The last residue with a shifted peak, Q134, is close to the C-terminal and it is the last one included in the IFNγΔ10 form of the protein used in measuring the reference spectrum. Q134 is not present in the crystallized structure of IFNγ used for structural analysis of NMR peak changes.

Out of the residues with shifted peaks, seven are positioned at the dimer interface of IFNγ: L34, E39, E40, D42, F82, V117 and A119. Two of these residues, E39 and D42, were also observed as significatly increasing in peak intensity upon addition of antibody in ID spectra. This strongly indicates that antibody 1 acts via disrupting the dimerization of IFNγ.

A remarkable observation concerning shifted peaks in the IFNγ-antibody1 spectrum, is that most changes in peak position and intensity are shifting the spectrum closer to the spectrum of IFNγ alone. That is, the spectrum of IFNγ together with antibody 1 resembles the reference spectrum in even more detail than the recorded spectrum for IFNγ alone. This tendency might be explained by the variety of oligomerization states of IFNγ.

The native state of IFNγ has been shown to be a symmetric homodimer. However, at any given point in time in a solution with IFNγ there will be molecules present in different states, in proportion to the probability of its oligomerization. In the characterization process of COS-1 expressed IFNγ with SDS-PAGE in certain conditions bands were observed around 75-80 kDa and even at 150 kDa, which would correspond approximately to the tetrameric and octameric form of IFNγ. A tetrameric structure for IFNγ has also been crystallized.

The COS-1 expressed IFNγ showed unusually strong tendency to maintain its dimeric state in reducing environment and to even oligomerize to higher states. SDS-PAGE experiments indicated that the presence of antibody in the IFNγ sample assisted depolymerization of the dimer in the gel, while IFNγ alone remained at least partly as a dimer.

This suggests that in the studied NMR sample of IFNγ there were more higher oligomerization states present than in the sample used for the reference spectrum. The observation that the IFNγ alone spectrum was in general rather broad supports this notion. If indeed the antibody disrupts oligomerization of IFNγ, it would be expected

that the IFNγ spectrum with the antibody would turn sharper and match the reference spectrum in more detail.

Figure 4.18 *A cartoon representation of a tetrameric form of IFNγ.*

5. ANALYSIS OF RESULTS

5.1 Expression of IFNγ receptor complex components

IFNγ and IFNγR1 were expressed in a transient transfection in COS-1 cells. The proteins were expressed in 0,01 mg scale, which was barely sufficient for basic characterization. For further conformational analysis with e.g. cysteine labeling or more extensive NMR spectrometry, a considerably larger amount of protein is required.

The expressed IFNγR1 was found to be resolved in dimeric form in SDS-PAGE, suggesting that the oligomerization of the receptor was unusually resistant to reducing conditions. In comparison, the purchased extracellular domain of IFNγR1 appeared on the SDS-PAGE as a monomer with a spread band due to varied glycosylation patterns in the sample. This indicates that IFNγR1 can be expressed in full length as a covalently bound dimer.

The binding of IFNγ-IFNγR1 complex was verified with high-affinity Streptavidin co-purification. The expressed Strep-tagged IFNγR1 was eluted from the column together with purchased His-tagged IFNγ. The following SDS-PAGE characterization of the elution fractions showed that IFNγ had bound to the receptor.

IFNγR2 was not expressed in COS-1 cells in amount applicable for structural analysis. For IFNγR2, however, a stably expressing in HEK-293 cell line was constructed. Previous results of protein expression in a tetracycline inducible HEK-293 cell line had reported yields in mg scale per 1 liter of cell suspension. In this experiment, the yield for IFNγR2 was only in 0,1 mg scale for the five suspension flasks in total. The yield for IFNγR2 in stable transfection was similar to the yield for IFNγR1 in transient transfection, which raised a doubt on the effectiveness of tetracycline induction.

After careful analysis of the stable expression protocol used and the applied description of tetracycline induction by Reeves et al. it was noticed that the plasmids used for expression had not been constructed for tetracycline induction. The pACMV promoter was missing the *tetO* operator sequence, which would initiate transcription of IFNγR2 in response to tetracycline treatment. The actual expression of IFNγR2 was thus not induced by tetracycline despite the treatment explaining the low yield. [64]

The lack of *tetO* promoter also explains the death of IFNγR1 transfected cells. Had the tetracycline inducible expression system been functional, the expression of the target protein would have been suppressed until the tetracycline treatment, thus enabling cell growth even if the target protein was toxic to host cells. In the absence of the *tetO* promoter, IFNγR1 was continuously expressed at a low level. While IFNγR2

transfected cells survived, the death of IFNγR1 transfected cells indicates that IFNγR1 is toxic to HEK-293 cells.

For the stable cell line construction, regular HEK-293 cell lines were used. In the tetracycline inducible expression of rhodopsin by Reeves et al. a N-acetylglucosaminyltransferase I –negative HEK-293 cell line was constructed for the expression. N-acetylglucosaminyltransferase I is responsible for synthesizing complex N-glycans and the lack of it prevents heavy posttranslational glycosylation of the target protein. [64]

SDS-PAGE characterization gave an apparent molecular weight of approximately 100 kDa for IFNγR2. Considering that the expressed IFNγ appeared partly as dimer and IFNγR1 appeared completely as a dimer in SDS-PAGE, the dimerization of IFNγR2 is not surprising. However, for a receptor monomer of 38 kDa, a molecular weight of approximately 80 kDa would be expected. The fact that N-glycosylation was not restricted could be responsible for the high observed molecular weight of IFNγR2.

5.2 Evidence for conformational changes in IFNγ

The identity of expressed IFNγ was first assessed with SDS-PAGE and then confirmed with an NMR analysis. The SDS-PAGE characterization showed clear duplet bands around at 19 kDa and 17 kDa, with 19 kDa being the expected molecular weight. In addition, a similar but weaker duplet pattern was observed at the molecular weight for IFNγ dimer, just above 35 kDa.

The cleavage of the Strep-tag could explain a narrow gap between the duplet lines, as the molecular weight of the Strep-tag is 1 kDa. Since SDS-PAGE can only be used as an approximate measure of molecular weight, the difference between the observed gap and the molecular weight of the Strep-tag is within the error margin.

A more likely explanation to the duplet nature of the IFNγ band is the partial proteolysis of glycosylation products. Mironova et al. have shown that IFNγ containing heavy glycosylation products undergoes both partial cleavage and dimerization during storage, both in 4°C and -20°C. [76]

All the expressed IFNγ was stored at -20°C between experiments, and the SDS-PAGE was run two weeks after purification. The SDS-PAGE results are thus consistent with previous research indicating that glycosylated IFNγ experiences partial proteolysis in thermally regulated conditions and covalent dimerization takes place for both full length and truncated forms.

The comparison of 2D NMR spectrum with an assigned reference spectrum confirmed the identity of the expressed protein as full length IFNγ. The general broadness of the recorded spectrum and the presence of several peaks not appearing in the reference are explained by the native heavy glycosylation of IFNγ and the consequent oligomerization of the protein.

Addition of a putative antibody in the IFNγ sample introduced both global and specific changes in the spectrum. Overall the intensity and the resolution of the spectrum improved. Most of the peaks that changed position or intensity were assigned to residues located at the dimer interface of IFNγ. The majority of observed changes in peak position upon antibody binding were directed towards closer correspondence with the position of reference peaks. This indicates that antibody binding changed the conformation of IFNγ towards its native state from the observed higher oligomerization states.

Overall it can be concluded that the experimented protocols for IFNγ and IFNγR1 expression in COS-1 cells, as well as IFNγR2 expression in HEK-293 cells, produced the desired target proteins as heavily glycosylated forms with a high tendency for covalent oligomerization. The oligomerization forms of IFNγ were disrupted by binding of the examined putative antibody 1. Unfortunately, although the complex formation of the expressed IFNγR1 with IFNγ was witnessed, the NMR study of the full receptor-ligand complex was not feasible with the produced amount of IFNγR1 and the available time for high-resolution NMR experiments.

6. CONCLUSIONS AND FUTURE WORK

In order to produce sufficiently protein for studying the interactions of the IFNγ-signaling complex in more detail, an inducible stably expressing cell line needs to be established. Inserting the *tetO* promoter in the recombinant DNA will allow for tetracycline regulated expression of target proteins.

Tetracycline regulation should make possible also the expression of IFNγR1, which is supposedly toxic to HEK-293 cells. Growing a large-scale suspension culture might require too much time for cells to survive, but producing a moderate amount of protein on petri dishes is likely to be feasible. In addition, possibilities to construct a glycosylation restricting cell line of HEK-293 cells should be looked for.

In this study, IFNγ and IFNγR1 were successfully expressed in amounts sufficient for characterization of the proteins and preliminary binding analysis. Co-purification of transiently expressed IFNγR1 to purchased human IFNγ confirmed binding of the expressed primary receptor to its ligand. Binding of expressed IFNγR2 to IFNγR1 can be assessed the same way by co-purifying it with non Strep-tagged IFNγR1 and IFNγ, both with each one separately and all three together. According to the current hypothesis, IFNγR2 should not bind to IFNγR1 or IFNγ alone, but only to the IFNγ-IFNγR1 complex.

Continuing further, JAK-1 can be co-purified with the whole ligand-receptor complex, with only one of the complex components carrying the Strep-tag. JAK-1 would then be expected to bind to the receptors only in presence of IFNγ, and only to both receptors together. The used protocol for Strep-Tactin purification would possibly require some modification for elution of such a large protein complex.

With more protein available, NMR analysis becomes a potent tool for studying the binding sites and conformational changes in IFNγ-receptor interactions and IFNγ-antibody interactions. The indicated putative binding sites of studied antibody 1 can be verified by introducing site-directed mutations to the locations of residues which are expected to participate in antibody binding, especially residues E39, E40, D42, F82, V117 and A119. If the indicated residues are indeed responsible for antibody binding, the NMR spectrum of expressed mutated IFNγ is then likely to remain the same with and without addition of antibody.

In the longer term, the signaling of the IFNγ receptors can be examined by expressing ^{15}N and ^{13}C labeled receptors and comparing NMR spectra for receptors alone and in complex with IFNγ. Although the binding sites of IFNγ to the extracellular side of IFNγR1 have already been unveiled, finding shifting in peaks representing intracellular amino acids of the receptor, would indicate coupling of conformational changes in the

intracellular and extracellular sides of the receptor upon ligand binding. This will help elucidate the allosteric signaling mechanism of enzyme-linked membrane receptors in general.

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APPENDIX I: Protocol for transformation of DNA in potent E-coli cells

- Place 0,5 µl of each DNA sample in a 1,5 ml tube with 35 µl of cells.
- Centrifuge shortly.
- Incubate cells for 30 min on ice and expose to a heat shock at 37°C for 45 s.
- Add 1 ml of LB medium and incubate cells for 1 hour in 37°C.
- Place 100 µl per plate of each resulting culture on plates with LB media containing Ampicillin.
- Let cells grow overnight in 37°C and pick 12-16 hours later.

APPENDIX II: Protocol for transfection of COS-1 and HEK-293 cells

Transient transfection of COS-1 cells with DEAE-dextran and chloroquine

Waking up cells:

- Wake up cells from liquid nitrogen by slowly (1 ml / 5 min) suspending them in DMEM[2].
- Centrifuge for 10 min in 3500 rpm at room temperature to diffuse remaining DMSO form cells.
- Re-suspend the cell pellet in 20 ml of DMEM on a 15 cm dish.
- Incubate at 37°C until approximately 80–90% confluence.

Splitting dishes with ratio from 1:3 to 1:10:

- Before cell splitting equilibrate all necessary solutions at 37ºC.
- Aspirate old medium from dishes.
- Wash dishes with 5 ml of PBS[1] or 2 ml of 1x trypsin (trypsin-EDTA in PBS).
- Add 2 ml of trypsin per dish and incubate at 37C for 2-5 min (check after 2 min if the cells are starting to detach from the dish, if not incubate more).
- Agitate dish until most cells have detached.
- Add 8 ml of DMEM_pf[2] per dish to inactivate trypsin.
- Remove cells from dish by pipetting up and down and transfer cells into appropriate number of new dishes.
- Add DMEM up to 20 ml per dish.
- Mix thoroughly by gently tilting the dish.
- Incubate at 37ºC until 90 % confluent.

Transient transfection:

- Aspirate old medium and wash dishes twice with 8 ml of DMEM_p[3].
- Add 12,5 µg of plasmid-DNA per dish in 10 ml of DMEM_p with 0,25 mg/ml DEAE-dextran and 0,1M Tris-HCl (pH 8).
- Incubate for 5-6 hours at 37ºC.
- Aspirate the solution and add 0,1 mM chloroquin in 15 ml of DMEM_p per dish.
- Incubate at 37ºC for 1,5-2 hours.
- Aspirate the chloroquin solution and wash twice with 8 ml of DMEM_p.

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[1] *PBS = Phosphate Buffered Saline containing 137 mM NaCl, 2,7 mM KCl, 10 mM Na_2HPO_4 and 1,76 mM KH_2PO_4 at pH 7,4.*

[2] *DMEM_pf = DMEM with 1% PenStrep and 10% Fetal Bovine Serum (FBS)*

[3] DMEM_p = DMEM with 1% PenStrep but no FBS

- Add 20 ml of DMEM_pf and incubate at 37ºC until the total incubation time from the addition of DNA is 55-60 hours.

Harvesting the transfected cells:

- Aspirate old medium and wash with 10 ml of PBS
- Add 5 ml of PBS per dish and scratch off the cells to sample tubes
- Flash freeze in liquid nitrogen and store in -20°C.

Stable transfection in HEK-293 cells

- Treat cells like in transient transfection with the difference of using DMEM-f12 medium.
- After transfection feed cells once every 2 days with DMEM-f12 containing 2% Geneticin (or another antibiotic depending on the resistance gene encoded in the recombinant DNA).
- Continue feeding until all non-transfected cells have died and a few resistant colonies remain on the dish.
- Pick resistant colonies taking care that those colonies are not cross contaminated.
- Expand picked colonies to grow monoclonal cultures expressing target protein in desired scale.

APPENDIX III: Protocol for purification of strep-tagged proteins with antibody affinity chromatography

- Solubilize protein extract in 1% DM in PBS.
- Mix on an end-over-end mixer in 4°c for 1h.
- Centrifuge in 4°C at 35000 rpm for 30 min.
- Remove supernatant and add 100 µl of Strep-beads per 150 µl of sample.
- End-over-end mix in 4°C for at least 4h (mixing overnight is possible).
- Apply supernatant in a separation column.
- Wash 10 times with 2 ml of 200 mM K-phosphate (pH8,0), with 0.05% DM.
- Wash 10 times with 2 ml of 100 mM Na-citrate (pH 8,0), with 0.05% DM.
- Elute in five separate 100 µl fractions of 2,5 mM Desthiobiotin in100 mM Na-citrate.

APPENDIX IV: Protocol for running an SDS-PAGE

Preparing the resolving gel:

- Clean glass plates carefully and assemble the glass sandwitch in the casting frame.
- Pour a little bit of water between glass plates to make sure assembly doesn't leak.
- Mix a 10 % polyacryleamide resolving gel in a 50 ml tube with
    - H_2O 9,0 ml
    - 1,5 M Tris, pH 8,8 5,6 ml
    - 10% SDS 225 µl
    - 30% PAA 7,5 ml
    - TeMed 25 µl
    - 10% APS 150 µl
- Mix quickly by vigorously shaking.
- Pour gel solution (~ 7 ml, leaving just enough space for the stacking gel and the comb) between glass plates directly after adding TeMed and APS leaving sufficiently space for stacking gel and comb.
- Pour a 2 mm layer of water on top.
- Let the gel polymerize for approximately 30 min, until remaining gel solution in the tube is polymerized.

Preparing the stacking gel:

- Mix a 4% polyacryleamide stacking gel in a 15 ml tube with
    - H_2O 2,9 ml
    - 0,5 M Tris, pH 6,8 1,25 ml
    - 10% SDS 50 µl
    - 30% PAA 650 µl
    - TeMed 50 µl
    - 10% APS 100 µl
- Pour stacking gel solution on top of resolving gel.
- Slide the comb was between the glass plates avoiding bubbles trapped under the comb.
- Let stacking gel polymerize for approximately 30 min.

Preparing the samples:

- Mix one part of sample loading buffer with two parts of protein sample, e.g. 30 µl of sample with 15 µl loading buffer.
- Centrifuge shortly to homogenize composition.
- If the properties of studied proteins allow it, bring to boil shortly to enhance denaturing

Running the gel:

- Place glass sandwhich with the polymerized gel in the clamping frame.
- Fill buffer chambers with running buffer.

- Remove the comb carefully while glass sandwitch is immersed in running buffer.
- Pipette samples to separate wells in the gel.
- Let electricity run at 100 V for 60 min.
- Remove the gel from between glass plates and stain or western blot to visualize bands.

Composition of 100 ml of sample loading buffer:

2M Tris-HCl, pH 6,8	7,5 ml
SDS	6,0 g
Glycerol	18 ml
Mercaptoethanol	3,0 ml
0,2% Bromophenol blue	6,0 ml
H_2O	65 ml

Composition of 1,0 l of running buffer:

Tris-base	12 g
Glycine	57,6 g
10% SDS	20 ml
H_2O	920 ml

Coomassie Blue staining:

- Wash gel 3 times for 5 min with 100 ml of water in gentle shaking.
- Stain gel for 1 h in 50 ml of Coomassie Blue stain in gentle shaking.
- Wash off stain with 2 times 1 h in 100 ml of water in gentle shaking.

APPENDIX V: Protocol for running a western blot with Strep-Tactin antibody

- Prepare transfer buffer and wash buffer in at least the following amounts:

250 ml of transfer buffer:

Tris-base	750 mg
Glycine	3,6 g
100% Methanol	50 ml

1,0 l of wash buffer:

Tris-base	6,0 g
NaCl	29 g
Tween 20	1,0 ml
HCl	3,5 ml

- Run an SDS-PAGE for samples as explained in appendix III (no staining is necessary).
- Soak two extra thick filter papers and a nitrocellulose membrane in the o buffer. Avoid touching the membrane.
- Place the nitrocellulose membrane on top of a filter paper, the gel on top of the membrane and a filter paper on top of all in a sandwich like assembly on a Transfer Cell.
- Let electricity run at 15 V for 60 min.
- Dissemble transfer cell and remove nitrocellulose membrane carefully without touching it.
- Incubate membrane in blocking solution of 2% milk protein in wash buffer for 60 min in gentle shaking.
- Wash membrane 3 times for 5 min with wash buffer.
- Incubate membrane for 60 min in a solution of 10 µl of Strep-Tactin antibody in 10 ml of wash buffer in gentle shaking.
- Wash membrane 4 times for 1 min in 10 ml of wash buffer.
- Mix 2 ml of chemiluminescence enhancer solution with stable peroxide buffer in 1:1 ratio and incubate the membrane in mixture for 1 min.
- Drain membrane without touching it and place between plastic wraps in a bioexposure cassette.
- Develop an autoradiograph film of the membrane to visualize bands.

APPENDIX VI: Sequences of IFNγ-signaling complex proteins

Amino acid sequences of IFNγ and related proteins from NCBI Protein Database. The transmembrane regions of IFNγR1 and IFNγR2 are underlined.

A. gi|20805896|gb|AAM28885.1| *Interferon-gamma* [Homo sapiens]

MQDPYVKEAENLKKYFNAGHSDVADNGTLFLGILKNWKEESDRKIMQSQIVSFY
FKLFKNFKDDQSIQKSVETIKEDMNVKFFNSNKKKRDDFEKLTNYSVTDLNVQR
KAIHELIQVMAELSPAAKTGKRTRSQMLFRGRRASQ

B. gi|5748602|emb|CAB53062.1| *Interferon gamma receptor 1* [Homo sapiens] (del 1-18)

EMGTADLGPSSVPTPTNVTIESYNMNPIVYWEYQIMPQVPVFTVEVKNYGVKNS
EWIDACINISHHYCNISDHVGDPSNSLWVRVKARVGQKESAYAKSEEFAVCRDG
KIGPPKLDIRKEEKQIMIDIFHPSVFVNGDEQEVDYDPETTCYIRVYNVYVRMN
GSEIQYKILTQKEDDCDEIQCQLAIPVSSLNSQYCVSAEGVLHVWGVTTEKSKE
VCITIFNSSIKG**SLWIPVVAALLLFLVLSLVFI**CFYIKKINPLKEKSIILPKSL
ISVVRSATLETKPESKYVSLITSYQPFSLEKEVVCEEPLSPATVPGMHTEDNPG
KVEHTEELSSITEVVTTEENIPDVVPGSHLTPIERESSSPLSSNQSEPGSIALN
SYHSRNCSESDHSRNGFDTDSSCLESHSSLSDSEFPPNNKGEIKTEGQELITVI
KAPTSFGYDKPHVLVDLLVDDSGKESLIGYRPTEDSKEFS

C. sp|P38484|INGR2_HUMAN| *Interferon gamma receptor 2*

MRPTLLWSLLLLLGVFAAAAAAPPDPLSQLPAPQHPKIRLYNAEQVLSWEPVAL
SNSTRPVVYQVQFKYTDSKWFTADIMSIGVNCTQITATECDFTAASPSAGFPMD
FNVTLRLRAELGALHSAWVTMPWFQHYRNVTVGPPENIEVTPGEGSLIIRFSSP
FDIADTSTAFFCYYVHYWEKGGIQQVKGPFRSNSISLDNLKPSRVYCLQVQAQL
LWNKSNIFRVGHLSNISCYETMADASTELQQ**VILISVGTFSLLSVLAGACFF**LV
LKYRGLIKYWFHTPPSIPLQIEEYLKDPTQPILEALDKDSSPKDDVWDSVSIIS
FPEKEQEDVLQTL

D. sp|P23458|JAK1_HUMAN| *Tyrosine-protein kinase JAK1*

MQYLNIKEDCNAMAFCAKMRSSKKTEVNLEAPEPGVEVIFYLSDREPLRLGSGE
YTAEELCIRAAQACRISPLCHNLFALYDENTKLWYAPNRTITVDDKMSLRLHYR
MRFYFTNWHGTNDNEQSVWRHSPKKQKNGYEKKKIPDATPLLDASSLEYLFAQG
QYDLVKCLAPIRDPKTEQDGHDIENECLGMAVLAISHYAMMKKMQLPELPKDIS
YKRYIPETLNKSIRQRNLLTRMRINNVFKDFLKEFNNKTICDSSVSTHDLKVKY
LATLETLTKHYGAEIFETSMLLISSENEMNWFHSNDGGNVLYYEVMVTGNLGIQ
WRHKPNVVSVEKEKNKLKRKKLENKHKKDEEKNKIREEWNNFSYFPEITHIVIK
ESVVSINKQDNKKMELKLSSHEEALSFVSLVDGYFRLTADAHHYLCTDVAPPLI
VHNIQNGCHGPICTEYAINKLRQEGSEEGMYVLRWSCTDFDNILMTVTCFEKSE
QVQGAQKQFKNFQIEVQKGRYSLHGSDRSFPSLGDLMSHLKKQILRTDNISFML
KRCCQPKPREISNLLVATKKAQEWQPVYPMSQLSFDRILKKDLVQGEHLGRGTR
THIYSGTLMDYKDDEGTSEEKKIKVILKVLDPSHRDISLAFFEAASMMRQVSHK
HIVYLYGVCVRDVENIMVEEFVEGGPLDLFMHRKSDVLTTPWKFKVAKQLASAL
SYLEDKDLVHGNVCTKNLLAREGIDSECGPFIKLSDPGIPITVLSRQECIERIP
WIAPECVEDSKNLSVAADKWSFGTTLWEICYNGEIPLKDKTLIEKERFYESRCR
PVTPSCKELADLMTRCMNYDPNQRPFFRAIMRDINKLEEQNPDIVSEKKPATEV
DPTHFEKRFLKRIRDLGEGHFGKVELCRYDPEGDNTGEQVAVKSLKPESGGNHI

ADLKKEIEILRNLYHENIVKYKGICTEDGGNGIKLIMEFLPSGSLKEYLPKNKN
KINLKQQLKYAVQICKGMDYLGSRQYVHRDLAARNVLVESEHQVKIGDFGLTKA
IETDKEYYTVKDDRDSPVFWYAPECLMQSKFYIASDVWSFGVTLHELLTYCDSD
SSPMALFLKMIGPTHGQMTVTRLVNTLKEGKRLPCPPNCPDEVYQLMRKCWEFQ
PSNRTSFQNLIEGFEALLK

E. sp|P42224|STAT1_HUMAN| *Signal transducer and activator of transcription 1-alpha/beta*

MSQWYELQQLDSKFLEQVHQLYDDSFPMEIRQYLAQWLEKQDWEHAANDVSFAT
IRFHDLLSQLDDQYSRFSLENNFLLQHNIRKSKRNLQDNFQEDPIQMSMIIYSC
LKEERKILENAQRFNQAQSGNIQSTVMLDKQKELDSKVRNVKDKVMCIEHEIKS
LEDLQDEYDFKCKTLQNREHETNGVAKSDQKQEQLLLKKMYLMLDNKRKEVVHK
IIELLNVTELTQNALINDELVEWKRRQQSACIGGPPNACLDQLQNWFTIVAESL
QQVRQQLKKLEELEQKYTYEHDPITKNKQVLWDRTFSLFQQLIQSSFVVERQPC
MPTHPQRPLVLKTGVQFTVKLRLLVKLQELNYNLKVKVLFDKDVNERNTVKGFR
KFNILGTHTKVMNMEESTNGSLAAEFRHLQLKEQKNAGTRTNEGPLIVTEELHS
LSFETQLCQPGLVIDLETTSLPVVVISNVSQLPSGWASILWYNMLVAEPRNLSF
FLTPPCARWAQLSEVLSWQFSSVTKRGLNVDQLNMLGEKLLGPNASPDGLIPWT
RFCKENINDKNFPFWLWIESILELIKKHLLPLWNDGCIMGFISKERERALLKDQ
QPGTFLLRFSESSREGAITFTWVERSQNGGEPDFHAVEPYTKKELSAVTFPDII
RNYKVMAAENIPENPLKYLYPNIDKDHAFGKYYSRPKEAPEPMELDGPKGTGYI
KTELISVSEVHPSRLQTTDNLLPMSPEEFDEVSRIVGSVEFDSMMNTV